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Method for the production of ketocarotenoids in petals of plants

Description

The present invention relates to a method for the production of ketocarotenoids by culturing plants which, in comparison with the wild type, show a modified ketolase activity in petals, to the genetically modified plants, and to their use as foods and feeds and for the production of ketocarotenoid extracts.

Carotenoids are synthesized *de novo* in bacteria, algae, fungi and plants. Ketocarotenoids, i.e. carotenoids comprising at least one keto group, such as, for example, astaxanthin, canthaxanthin, echinenone, 3-hydroxyechinenone, 3'-hydroxyechinenone, adonirubin and adonixanthin are natural antioxidants and pigments which are produced by some algae and microorganisms as secondary metabolites.

Owing to their color-imparting properties, the ketocarotenoids, and in particular astaxanthin, are used as pigmenting auxiliaries in animal nutrition, in particular in trout, salmon and shrimp farming.

Currently, astaxanthin is largely produced synthetically by chemical methods. Natural ketocarotenoids, such as, for example, natural astaxanthin, are currently obtained in small amounts by biotechnological methods by culturing algae, for example Haematococcus pluvialis, or by fermenting microorganisms which have been optimized by genetic engineering, followed by isolation.

An economical biotechnological method for the production of natural ketocarotenoids is therefore of great importance.

WO 00/32788 discloses that certain carotenoid ratios in Tagetes petals can be influenced by combining the overexpression of carotenoid biosynthesis genes and antisense methods.

WO 98/18910 describes the synthesis of ketocarotenoids in nectar glands of tobacco flowers by introducing a ketolase gene into tobacco.

WO 01/20011 describes a DNA construct for the production of ketocarotenoids, in particular astaxanthin, in the seeds of oilseed plants such as oilseed rape, sunflower, soybean and mustard, using a seed-specific promoter and a ketolase from

Haematococcus.

While the methods disclosed in WO 98/18910 and WO 01/20011 yield genetically modified plants with a ketocarotenoid content in the specific tissues, they have the disadvantage that the level of the ketocarotenoid content and the purity, in particular with regard to astaxanthin, is as yet unsatisfactory.

The invention was therefore based on the object of providing an alternative method for the production of ketocarotenoids by culturing plants, or of providing further transgenic plants which produce ketocarotenoids, which have the optimized characteristics, such as, for example, a higher ketocarotenoid content, and which do not suffer from the above-described disadvantage of the prior art.

Accordingly, there has been found a method for the production of ketocarotenoids by culturing genetically modified plants which, in comparison with the wild type, show a modified ketolase activity in petals.

Apart from a few exceptions such as, for example, Adonis, plants, in particular the petals, contain carotenoids, but no ketocarotenoids. This is why, as a rule, the petals of wild type plants show no ketolase activity.

This is why, in one embodiment of the method according to the invention, the starting plants used are plants which show a ketolase activity in petals even as the wild type, such as, for example, Adonis. In this embodiment, the genetic modification brings about an increase of the ketolase activity in petals.

Ketolase activity is understood as meaning the enzyme activity of a ketolase.

A ketolase is understood as meaning a protein with the enzymatic activity of introducing a keto group at the optionally substituted β-ionone ring of carotenoids.

In particular, a ketolase is understood as meaning a protein with the enzymatic activity of converting β-carotene into canthaxanthin.

Accordingly, ketolase activity is understood as meaning the amount of β-carotene converted, or the amount of canthaxanthin formed, by the protein ketolase within a certain period of time.

Thus, in the case of an increased ketolase activity in comparison with the wild type, the amount of β-carotene converted, or the amount of canthaxanthin formed, by the protein ketolase within a certain period of time is increased in comparison with the wild type.

By preference, this increase of the ketolase activity amounts to at least 5%, furthermore preferably at least 20%, furthermore preferably at least 50%, furthermore preferably at least 100%, more preferably at least 300%, even more preferably at least 500%, in particular at least 600%, of the ketolase activity of the wild type.

In accordance with the invention, the term "wild type" is understood as meaning the corresponding non-genetically-modified starting plant.

Depending on the context, the term "plant" can be understood as meaning the starting plant (wild type), or a genetically modified plant according to the invention or both.

Preferably, and in particular in those cases where the plant or the wild type cannot be identified unambiguously, "wild type" for increasing or generating the ketolase activity, for the increase of the hydroxylase activity described hereinbelow, for the increase of the β-cyclase activity described hereinbelow, for the increase of the HMG-CoA reductase activity described hereinbelow, for the increase of the (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity described hereinbelow, for the increase of the 1-deoxy-D-xylose-5-phosphate synthase activity described hereinbelow, for the increase of the 1-deoxy-D-xylose-5-phosphate reductoisomerase activity described hereinbelow, for the increase of the isopentenyl-diphosphate Δ-isomerase activity described hereinbelow, for the increase of the geranyl-diphosphate synthase activity described hereinbelow, for the increase of the farnesyl-diphosphate synthase activity described hereinbelow, for the increase of the geranylgeranyl-diphosphate synthase activity described hereinbelow, for the increase of the phytoene synthase activity described hereinbelow, for the increase of the phytoene desaturase activity described hereinbelow, for the increase of the zeta-carotene desaturase activity described hereinbelow, for the increase of the crtISO activity described hereinbelow, for the increase of the FtsZ activity described hereinbelow, for the increase of the MinD activity described hereinbelow, for the reduction of the ε-cyclase activity described hereinbelow and for the reduction of the endogenous β-hydroxylase activity described

herienbelow and for the increase of the ketocarotenoid content is in each case understood as meaning a reference plant.

For plants which already show a ketolase activity in petals as the wild type, this reference plant is by preference *Adonis aestivalis*, *Adonis flammeus* or *Adonis annuus*, especially preferably *Adonis aestivalis*.

For plants which show no ketolase activity in petals as the wild type, this reference plant is preferably Tagetes erecta, Tagetes patula, Tagetes lucida, Tagetes pringlei, Tagetes palmeri, Tagetes minuta or Tagetes campanulata, especially preferably *Tagetes erecta*.

The ketolase activity in genetically modified plants of the invention and in wild type or reference plants is determined under the following conditions:

The ketolase activity in plant material is determined by a method similar to that of Frazer et al., (J. Biol. Chem. 272(10): 6128-6135, 1997). The ketolase activity in plant extracts is determined using the substrates beta-carotene and canthaxanthin in the presence of lipid (soya lecithin) and detergent (sodium cholate). Substrate/product ratios from the ketolase assays are determined by HPLC.

The ketolase activity can be increased in various ways, for example by eliminating inhibiting regulatory mechanisms at the translation and protein level, or by increasing the gene expression of a nucleic acid encoding a ketolase in comparison with the wild type, for example by inducing the ketolase gene by activators or by introducing, into the plant, nucleic acids encoding a ketolase.

In accordance with this embodiment according to the invention, increasing the gene expression of a nucleic acid encoding a ketolase is also understood as meaning the manipulation of the expression of the plants' homologous endogenous ketolases. This can be achieved for example by modifying the promoter DNA sequence for ketolase-encoding genes. Such a modification which results in a modified or, with preference increased, expression rate of at least one endogenous ketolase gene can be effected by deletion or insertion of DNA sequences.

As described above, it is possible to modify the expression of at least one endogenous ketolase by applying exogenous stimuli. This can be carried out by specific physiological conditions, i.e. by

the application of foreign substances.

Moreover, an increased expression of at least one endogenous ketolase gene can be achieved by a regulator protein which does not occur in the wild-type plant, or which is modified, interacting with the promoter of these genes.

Such a regulator can constitute a chimeric protein which consists of a DNA binding domain and a transcription activator domain such as described, for example, in WO 96/06166.

In a preferred embodiment, increasing the ketolase activity in comparison with the wild type is effected by increasing the gene expression of a nucleic acid encoding a ketolase.

In a further preferred embodiment, increasing the gene expression of a nucleic acid encoding a ketolase is effected by introducing, into the plant, nucleic acids which encode ketolases.

In this embodiment, there is thus at least one further ketolase gene present in the transgenic plants according to the invention in comparison with the wild type. In this embodiment, the genetically modified plant according to the invention, accordingly, has at least one exogenous (= heterologous) nucleic acid encoding a ketolase, or at least two endogenous nucleic acids encoding a ketolase.

In another preferred embodiment of the method according to the invention, the starting plants used are plants which, as the wild type, show no ketolase activity in petals, such as, for example, *tomato, marigold, Tagetes erecta, Tagetes lucida, Tagetes minuta, Tagetes pringlei, Tagetes palmeri* and *Tagetes campanulata*.

In this preferred embodiment, the genetic modification generates the ketolase activity in petals. In this preferred embodiment, the genetically modified plant according to the invention thus has, in comparison with the genetically nonmodified wild type, a ketolase activity in petals and is thus preferably capable of transgenically expressing a ketolase in petals.

In this preferred embodiment, generating the gene expression of a nucleic acid encoding a ketolase takes place analogously to the above-described increase of the gene expression of a nucleic acid encoding a ketolase, preferably by introducing, into the starting plant, nucleic acids which encode ketolases.

To this end, it is possible, in principle, that any ketolase gene, that is to say any nucleic acid which encodes a ketolase, can be used in both these embodiments.

All the nucleic acids mentioned in the description can be for example an RNA, DNA or cDNA sequence.

In the case of genomic ketolase sequences from eukaryotic sources, which comprise introns, nucleic acid sequences which are preferably to be used are, in the event that the host plant is not capable, or cannot be made capable, of expressing the ketolase in question, ready-processed nucleic acids such as the corresponding cDNAs.

Examples of nucleic acids encoding a ketolase, and the corresponding ketolases, which can be used in the method according to the invention are, for example, sequences from

Haematoccus pluvialis, in particular from Haematoccus pluvialis Flotow em. Wille (Accession NO: X86782; nucleic acid: SEQ ID NO: 1, protein SEQ ID NO: 2),

Haematoccus pluvialis, NIES-144 (Accession NO: D45881; nucleic acid: SEQ ID NO: 3, protein SEQ ID NO: 4),

Agrobacterium aurantiacum (Accession NO: D58420; nucleic acid: SEQ ID NO: 5, protein SEQ ID NO: 6),

Alicaligenes spec. (Accession NO: D58422; nucleic acid: SEQ ID NO: 7, protein SEQ ID NO: 8),

Paracoccus marcusii (Accession NO: Y15112; nucleic acid: SEQ ID NO: 9, protein SEQ ID NO: 10).

Synechocystis sp. strain PC6803 (Accession NO: NP442491; nucleic acid: SEQ ID NO: 11, protein SEQ ID NO: 12).

Bradyrhizobium sp. (Accession NO: AF218415; nucleic acid: SEQ ID NO: 13, protein SEQ ID NO: 14).

Nostoc sp. strain PCC7120 (Accession NO: AP003592, BAB74888; nucleic acid: SEQ ID NO: 15, protein SEQ ID NO: 16).

Haematococcus pluvialis
(Accession NO: AF534876, AAN03484; nucleic acid: SEQ ID NO: 81, protein : SEQ ID NO: 82)

Paracoccus sp. MBIC1143
(Accession NO: D58420, P54972; nucleic acid: SEQ ID NO: 83, protein : SEQ ID NO: 84)

Brevundimonas aurantiaca
(Accession NO: AY166610, AAN86030; nucleic acid: SEQ ID NO: 85, protein : SEQ ID NO: 86)

Nodularia spumigena NSOR10
(Accession NO: AY210783, AAO64399; nucleic acid: SEQ ID NO: 87, protein : SEQ ID NO: 88)

Nostoc punctiforme ATCC 29133
(Accession NO: NZ_AABC01000195, ZP_00111258; nucleic acid: SEQ ID NO: 89, protein : SEQ ID NO: 90)

Nostoc punctiforme ATCC 29133
(Accession NO: NZ_AABC01000196; nucleic acid: SEQ ID NO: 91, protein : SEQ ID NO: 92)

Deinococcus radiodurans R1
(Accession NO: E75561, AE001872; nucleic acid: SEQ ID NO: 93, protein : SEQ ID NO: 94)

Further natural examples of ketolases and ketolase genes which can be used in the method according to the invention can be found readily for example from various organisms whose genomic sequence is known by carrying out alignments of the amino acid sequences or of the corresponding backtranslated nucleic acid sequences from databases with the above-described sequences, and in particular with the sequences SEQ ID NO: 2 and/or 16 and/or 90 and/or 92.

Further natural examples of ketolases and ketolase genes can furthermore be found readily from different organisms whose genomic sequence is not known by using hybridization techniques in the manner known per se, starting from the above-described nucleic acid sequences, in particular starting from the sequences SEQ ID NO: 2 and/or 16 and/or 90 and/or 92.

The hybridization can be carried out under moderate (low-stringency) or, preferably under stringent (high-stringency) conditions.

Such hybridization conditions are described, for example, in Sambrook, J., Fritsch, E.F., Maniatis, T., in: Molecular Cloning (A Laboratory Manual), 2nd Edition, Cold Spring Harbor Laboratory

Press, 1989, pages 9.31-9.57 or in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6.

For example, the conditions during the washing step can be selected from the range of conditions delimited by those with less stringency (with 2X SSC at 50°C) and those with high stringency (with 0.2X SSC at 50°C, preferably at 65°C) (20X SSC: 0.3 M sodium citrate, 3 M sodium chloride, pH 7.0).

Moreover, the temperature during the washing step can be increased from moderate conditions at room temperature, 22°C, to stringent conditions at 65°C.

Both parameters, salt concentration and temperature can be varied simultaneously, or else one of the two parameters can be kept constant, while only the other one is varied. Also, denaturing agents such as, for example, formamide or SDS can be employed during the hybridization step. In the presence of 50% formamide, the hybridization is preferably carried out at 42°C.

Some examples of conditions for hybridization and washing step are shown hereinbelow:

(1) hybridization conditions with, for example,

(i) 4X SSC at 65°C, or

(ii) 6X SSC at 45°C, or

(iii) 6X SSC at 68°C, 100 mg/ml denatured fish sperm DNA, or

(iv) 6X SSC, 0.5% SDS, 100 mg/ml denatured, fragmented salmon sperm DNA at 68°C, or

(v) 6X SSC, 0.5% SDS, 100 mg/ml denatured, fragmented salmon sperm DNA, 50% formamide at 42°C, or

(vi) 50% formamide, 4X SSC at 42°C, or

(vii) 50% (vol/vol) formamide, 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 50 mM sodium phosphate buffer pH 6.5, 750 mM NaCl, 75 mM sodium citrate at 42°C, or

(viii) 2X or 4X SSC at 50°C (moderate conditions), or

(ix) 30 to 40% formamide, 2X or 4X SSC at 42°C (moderate conditions).

(2) washing steps for in each case 10 minutes, with, for example,

(i) 0.015 M NaCl/0.0015 M sodium citrate/0.1% SDS at 50°C, or

(ii) 0.1X SSC at 65°C, or

(iii) 0.1X SSC, 0.5% SDS at 68°C, or

(iv) 0.1X SSC, 0.5% SDS, 50% formamide at 42°C, or

(v) 0.2X SSC, 0.1% SDS at 42°C, or

(vi) 2X SSC at 65°C (moderate conditions).

In a preferred embodiment of the methods according to the invention, nucleic acids are introduced which encode a protein comprising the amino acid sequence SEQ ID NO: 2 or a sequence derived from this sequence by substitution, insertion or deletion of amino acids which has at least 20%, by preference at least 30%, more preferably at least 40%, more preferably at least 50%, more preferably at least 60%, more preferably at least 70%, more preferably at least 80%, especially preferably at least 90% identity at the amino acid level with the sequence SEQ ID NO: 2 and which has the enzymatic characteristic of a ketolase.

This may take the form of a natural ketolase sequence which can be found from other organisms as described above by alignment of the sequences, or else an artificial ketolase sequence which has been modified starting from the sequence SEQ ID NO: 2 by artificial variation, for example by substitution, insertion or deletion of amino acids.

In a further, preferred embodiment of the methods according to the invention, nucleic acids are introduced which encode a protein comprising the amino acid sequence SEQ ID NO: 16 or a sequence derived from this sequence by substitution, insertion or deletion of amino acids which has at least 20%, by preference at least 30%, more preferably at least 40%, more preferably at least 50%, more preferably at least 60%, more preferably at least 70%, more preferably at least 80%, especially preferably at least 90% identity at the amino acid level with the sequence SEQ ID NO: 16 and which has the enzymatic characteristic of a ketolase.

This may take the form of a natural ketolase sequence which can be found from other organisms as described above by alignment of the sequences, or else an artificial ketolase sequence which has been modified starting from the sequence SEQ ID NO: 16 by artificial variation, for example by substitution, insertion or deletion of amino acids.

In a further, preferred embodiment of the methods according to the invention, nucleic acids are introduced which encode a protein comprising the amino acid sequence SEQ ID NO: 90 or a sequence derived from this sequence by substitution, insertion or deletion of amino acids which has at least 20%, by preference at least 30%, more preferably at least 40%, more preferably at least 50%, more preferably at least 60%, more preferably at least 70%, more preferably at least 80%, especially preferably at least 90% identity at the amino acid level with the sequence SEQ ID NO: 90 and which has the enzymatic characteristic of a ketolase.

This may take the form of a natural ketolase sequence which can be found from other organisms as described above by alignment of the sequences, or else an artificial ketolase sequence which has been modified starting from the sequence SEQ ID NO: 90 by artificial variation, for example by substitution, insertion or deletion of amino acids.

In a further, preferred embodiment of the methods according to the invention, nucleic acids are introduced which encode a protein comprising the amino acid sequence SEQ ID NO: 92 or a sequence derived from this sequence by substitution, insertion or deletion of amino acids which has at least 20%, by preference at least 30%, more preferably at least 40%, more preferably at least 50%, more preferably at least 60%, more preferably at least 70%, more preferably at least 80%, especially preferably at least 90% identity at the amino acid level with the sequence SEQ ID NO: 92 and which has the enzymatic characteristic of a ketolase.

This may take the form of a natural ketolase sequence which can be found from other organisms as described above by alignment of the sequences, or else an artificial ketolase sequence which has been modified starting from the sequence SEQ ID NO: 92 by artificial variation, for example by substitution, insertion or deletion of amino acids.

In the description, the term "substitution" is understood as meaning the replacement of one or more amino acids by one or more amino acids. Substitutions which are preferably carried out are what are known as conservative substitutions, where the replaced

amino acid has a similar property to the original amino acid, for example substitution of Glu by Asp, Gln by Asn, Val by Ile, Leu by Ile, Ser by Thr.

Deletion is the replacement of an amino acid by a direct bond. Preferred positions for deletion are the termini of the polypeptide and the linkages between the individual protein domains.

Insertions are introductions of amino acids into the polypeptide chain, where a direct bond is formally replaced by one or more amino acids.

Identity between two proteins is understood as meaning the identity of the amino acids over in each case the entire protein length, in particular the identity which is calculated by comparison with the aid of the Lasergene software from DNASTAR, inc. Madison, Wisconsin (USA) using the Clustal method (Higgins DG, Sharp PM. Fast and sensitive multiple sequence alignments on a microcomputer. Comput Appl. Biosci. 1989 Apr;5(2):151-1), setting the following parameters:

Multiple alignment parameter:	
Gap penalty	10
Gap length penalty	10
Pairwise alignment parameter:	
K-tuple	1
Gap penalty	3
Window	5
Diagonals saved	5

A protein which has at least 20% identity at the amino acid level with a certain sequence is, accordingly, understood as meaning a protein which, upon comparison of its sequence with the particular sequence, in particular by the above program algorithm with the above parameter set, has at least 20% identity.

Accordingly, a protein which has at least 20% identity at the amino acid level with the sequence SEQ ID NO: 2 or 16 or 90 or 92 is, accordingly, understood as meaning a protein which, upon comparison of its sequence with the sequence SEQ ID NO: 2 or 16 or 90 or 92, in particular by the above program algorithm with the above parameter set, has at least 20% identity.

Suitable nucleic acid sequences are obtainable, for example, by backtranslation of the polypeptide sequence according to the genetic code.

Codons which are preferably used for this purpose are those which are used frequently in accordance with the plant-specific codon usage. The codon usage can be determined readily with the aid of computer evaluations of other, known genes of the organisms in question.

In an especially preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 1 is introduced into the plant.

In a further especially preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 15 is introduced into the plant.

In a further especially preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 89 is introduced into the plant.

In a further especially preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 91 is introduced into the plant.

All the abovementioned ketolase genes can furthermore be generated in the known manner by chemical synthesis, starting with the nucleotide units, such as, for example, by fragment condensation of individual overlapping, complementary nucleic acid units of the double helix. Oligonucleotides can be synthesized chemically in the known manner for example by the phosphoamidite method (Voet, Voet, 2nd Edition, Wiley Press New York, pp. 896-897). The annealing of synthetic oligonucleotides and filling in of gaps by means of the Klenow fragment of the DNA polymerase and ligation reactions, and general cloning methods, are described in Sambrook et al. (1989), Molecular cloning: A laboratory manual, Cold Spring Harbor Laboratory Press.

In an especially preferred embodiment of the method according to the invention, genetically modified plants which show the highest expression rate of a ketolase in flowers are used.

This is preferably achieved by the gene expression of the ketolase taking place under the control of a flower-specific promoter. For example, the above-described nucleic acids as described hereinbelow in detail are introduced into the plant in

a nucleic acid construct in functional linkage with a flower-specific promoter.

In accordance with the invention, plants are preferably understood as meaning plants which, as the wild type, have chromoplasts in petals. Further preferred plants additionally have, as the wild type, carotenoids, in particular β-carotene, zeaxanthin, neoxanthin, violaxanthin or lutein, in the petals. Further preferred plants have, as the wild type, additionally a hydroxylase activity in the petals.

Hydroxylase activity is understood as meaning the enzyme activity of a hydroxylase.

A hydroxylase is understood as meaning a protein with the enzymatic activity of introducing a hydroxyl group at the optionally substituted β-ionone ring of carotenoids.

In particular, a hydroxylase is understood as meaning a protein with the enzymatic activity of converting β-carotene into zeaxanthin or cantaxanthin into astaxanthin.

Accordingly, hydroxylase activity is understood as meaning the amount of β-carotene or cantaxanthin converted, or the amount of zeaxanthin or astaxanthin formed, by the protein hydroxylase within a certain period of time.

Especially preferred plants are plants selected from the families Ranunculaceae, Berberidaceae, Papaveraceae, Cannabaceae, Rosaceae, Fabaceae, Linaceae, Vitaceae, Brassicaceae, Cucurbitaceae, Primulaceae, Caryophyllaceae, Amaranthaceae, Gentianaceae, Geraniaceae, Caprifoliaceae, Oleaceae, Tropaeolaceae, Solanaceae, Scrophulariaceae, Asteraceae, Liliaceae, Amaryllidaceae, Poaceae, Orchidaceae, Malvaceae, Illiaceae or Lamiaceae.

Very especially preferred plants are selected from the group of the plant genera *Marigold, Tagetes errecta, Tagetes patula, Acacia, Aconitum, Adonis, Arnica, Aquilegia, Aster, Astragalus, Bignonia, Calendula, Caltha, Campanula, Canna, Centaurea, Cheiranthus, Chrysanthemum, Citrus, Crepis, Crocus, Curcurbita, Cytisus, Delonia, Delphinium, Dianthus, Dimorphotheca, Doronicum, Eschscholtzia, Forsythia, Fremontia, Gazania, Gelsemium, Genista, Gentiana, Geranium, Gerbera, Geum, Grevillea, Helenium, Helianthus, Hepatica, Heracleum, Hisbiscus, Heliopsis, Hypericum, Hypochoeris, Impatiens, Iris, Jacaranda, Kerria, Laburnum, Lathyrus, Leontodon, Lilium, Linum, Lotus, Lycopersicon,*

Lysimachia, Maratia, Medicago, Mimulus, Narcissus, Oenothera, Osmanthus, Petunia, Photinia, Physalis, Phyteuma, Potentilla, Pyracantha, Ranunculus, Rhododendron, Rosa, Rudbeckia, Senecio, Silene, Silphium, Sinapsis, Sorbus, Spartium, Tecoma, Torenia, Tragopogon, Trollius, Tropaeolum, Tulipa, Tussilago, Ulex, Viola or *Zinnia*, especially preferably selected from the group of the plant genera Marigold, Tagetes erecta, Tagetes patula, Lycopersicon, Rosa, Calendula, Physalis, Medicago, Helianthus, Chrysanthemum, Aster, Tulipa, Narcissus, Petunia, Geranium, Tropaeolum or Adonis.

In a preferred embodiment, plants are cultured which additionally show an increased hydroxylase activity and/or β-cyclase activity in comparison with the wild type.

Hydroxylase activity is understood as meaning the enzyme activity of a hydroxylase.

A hydroxylase is understood as meaning a protein with the enzymatic activity of introducing a hydroxyl group at the optionally substituted β-ionone ring of carotenoids.

In particular, a hydroxylase is understood as meaning a protein with the enzymatic activity of converting β-carotene into zeaxanthin or cantaxanthin into astaxanthin.

Accordingly, hydroxylase activity is understood as meaning the amount of β-carotene or cantaxanthin converted, or the amount of zeaxanthin or astaxanthin formed, by the protein hydroxylase within a certain period of time.

Thus, in the case of a hydroxylase activity which is increased in comparison with the wild type, the converted amount of β-carotene or cantaxanthin, or the amount of zeaxanthin or astaxanthin formed, by the protein hydroxylase is increased within a certain period of time in comparison with the wild type.

This increase of the hydroxylase activity amounts by preference to at least 5%, furthermore preferably at least 20%, furthermore preferably at least 50%, furthermore preferably at least 100%, more preferably at least 300%, even more preferably at least 500%, in particular at least 600% of the hydroxylase activity of the wild type.

The "endogenous β-hydroxylase" described hereinbelow is understood as meaning the plant's homologous, endogenous hydroxylase. The

activity is determined analogously.

β-Cyclase activity is understood as meaning the enzyme activity of a β-cyclase.

A β-cyclase is understood as meaning a protein with the enzymatic activity of converting a terminal, linear residue of lycopene into a β-ionone ring.

In particular, a β-cyclase is understood as meaning a protein with the enzymatic activity of converting γ-carotene into β-carotene.

Accordingly, β-cyclase activity is understood as meaning the amount of γ-carotene converted, or the amount of β-carotene formed, by the protein β-cyclase within a certain period of time.

Thus, in the case of an increased β-cyclase activity in comparison with the wild type, the amount of γ-carotene converted, or the amount of β-carotene formed, by the protein β-cyclase within a certain period of time is increased in comparison with the wild type.

This increase of the β-cyclase activity amounts by preference to at least 5%, furthermore preferably at least 20%, furthermore preferably at least 50%, furthermore preferably at least 100%, more preferably at least 300%, even more preferably at least 500%, in particular at least 600% of the β-cyclase activity of the wild type.

The hydroxylase activity in genetically modified plants according to the invention and in wild-type, or reference, plants is preferably determined under the following conditions:

The hydroxylase activity is determined *in vitro* by the method of Bouvier et al. (Biochim. Biophys. Acta 1391 (1998), 320-328). Ferredoxin, ferredoxin-NADP oxidoreductase, catalase, NADPH and beta-carotene together with mono- and digalactosylglycerides are added to a certain amount of plant extract.

The hydroxylase activity is especially preferably determined by the method of Bouvier, Keller, d'Harlingue and Camara (Xanthophyll biosynthesis: molecular and functional characterization of carotenoid hydroxylases from pepper fruits (Capsicum annuum L.; Biochim. Biophys. Acta 1391 (1998), 320-328) under the following conditions:

The *in-vitro* assay is carried out in a volume of 0.250 ml. The mixture comprises 50 mM potassium phosphate (pH 7.6), 0.025 mg spinach ferredoxin, 0.5 units spinach ferredoxin-NADP+ oxidoreductase, 0.25 mM NADPH, 0.010 mg beta-carotene (emulsified in 0.1 mg Tween 80), 0.05 mM of a mixture of mono- and digalactosylglycerides (1:1), 1 unit catalase, 200 mono- and digalactosylglycerides (1:1), 0.2 mg bovine serum albumin and plant extract in different volumes. The reaction mixture is incubated for 2 hours at 30°C. The reaction products are extracted with organic solvents such as acetone or chloroform/methanol (2:1) and determined by means of HPLC.

The β-cyclase activity in genetically modified plants according to the invention and in wild-type, or reference, plants is preferably determined under the following conditions:

The β-cyclase activity is determined *in vitro* by the method of Fraser and Sandmann (Biochem. Biophys. Res. Comm. 185(1) (1992) 9-15). The following are added to a certain amount of plant extract: potassium phosphate to act as buffer (pH 7.6), lycopene to act as substrate, stromaprotein from Capsicum, NADP+, NADPH and ATP.

The hydroxylase activity is especially preferably carried out by the method of Bouvier, d'Harlingue and Camara (Molecular Analysis of carotenoid cyclae inhibition; Arch. Biochem. Biophys. 346(1) (1997) 53-64) under the following conditions:

The in-vitro assay is carried out in a volume of 250 µl. The mixture comprises 50 mM potassium phosphate (pH 7.6), different amounts of plant extract, 20 nM lycopene, 250 µg of chromoplastidial stromaprotein from Capsicum, 0.2 mM NADP+, 0.2 mM NADPH and 1 mM ATP. NADP/NADPH and ATP are dissolved in 10 µl of ethanol together with 1 mg of Tween 80 immediately prior to addition to the incubation medium. After a reaction time of 60 minutes at 30°C, the reaction is quenched by addition of chloroform/methanol (2:1). The reaction products which are extracted in chloroform are analyzed by means of HPLC.

An alternative assay in which radioactive substrate is used is described in Fraser and Sandmann (Biochem. Biophys. Res. Comm. 185(1) (1992) 9-15).

Increasing the hydroxylase activity and/or β-cyclase activity can be effected in various ways, for example by eliminating inhibiting regulatory mechanisms at the expression and protein level, or by increasing the gene expression of nucleic acids

encoding a hydroxylase and/or nucleic acids encoding a β-cyclase in comparison with the wild type.

Increasing the gene expression of the nucleic acids encoding a hydroxylase and/or increasing the gene expression of the nucleic acid encoding a β-cyclase in comparison with the wild type can likewise be effected in various ways, for example by inducing the hydroxylase gene and/or β-cyclase gene by activators, or by introducing one or more hydroxylase gene copies and/or β-cyclase gene copies, i.e. by introducing, into the plant, at least one nucleic acid encoding a hydroxylase and/or at least one nucleic acid encoding an ε-cyclase.

Increasing the gene expression of a nucleic acid encoding a hydroxylase and/or β-cyclase is also understood as meaning, in accordance with the invention, the manipulation of the expression of the plants' homologous, endogenous hydroxylase and/or β-cyclase.

In certain plants, in which the biosynthesis relies heavily on the α-carotenoid pathway, such as, for example, plants of the genus Tagetes, it is advantageous to reduce the endogenous β-hydroxylase activity and to increase the activity of exogenous hydroxylases.

This can be achieved for example by modifying the promoter DNA sequence of genes encoding hydroxylases and/or β-cyclases. Such a modification, which results in an increased expression rate of the gene, can be effected for example by the deletion or insertion of DNA sequences.

As described above, it is possible to modify the expression of the endogenous hydroxylase and/or β-cyclase by applying exogenous stimuli. This can be effected by specific physiological conditions, i.e. by the application of foreign substances.

Moreover, a modified, or increased, expression of an endogenous hydroxylase and/or β-cyclase gene can be achieved by a regulator protein which does not occur in the untransformed plant interacting with the promoter of this gene.

Such a regulator can be a chimeric protein which consists of a DNA binding domain and a transcription activator domain, as described, for example, in WO 96/06166.

In a preferred embodiment, the gene expression of a nucleic acid encoding a hydroxylase and/or increasing the gene expression of a

nucleic acid encoding a β-cyclase is effected by introducing, into the plant, at least one nucleic acid encoding a hydroxylase and/or by introducing, into the plant, at least one nucleic acid encoding a β-cyclase.

In principle, any hydroxylase gene, or any β-cyclase gene, i.e. any nucleic acid which encodes a hydroxylase and any nucleic acid which encodes a β-cyclase can be used for this purpose.

In the case of genomic hydroxylase or β-cyclase nucleic acid sequences from eukaryotic sources, which comprise introns, it is preferred to use ready-processed nucleic acid sequences, such as the corresponding cDNAs, in the event that the host plant is not capable, or cannot be made capable, of expressing the hydroxylase or β-cyclase in question.

Examples of a hydroxylase gene are:
a nucleic acid encoding a hydroxylase from Haematococcus pluvialis, Accession AX038729, WO 0061764); (nucleic acid: SEQ ID NO: 17, protein: SEQ ID NO: 18),

and hydroxylases of the following Accession numbers:

|emb|CAB55626.1, CAA70427.1, CAA70888.1, CAB55625.1, AF499108_1, AF315289_1, AF296158_1, AAC49443.1, NP_194300.1, NP_200070.1, AAG10430.1, CAC06712.1, AAM88619.1, CAC95130.1, AAL80006.1, AF162276_1, AAO53295.1, AAN85601.1, CRTZ_ERWHE, CRTZ_PANAN, BAB79605.1, CRTZ_ALCSP, CRTZ_AGRAU, CAB56060.1, ZP_00094836.1, AAC44852.1, BAC77670.1, NP_745389.1, NP_344225.1, NP_849490.1, ZP_00087019.1, NP_503072.1, NP_852012.1, NP_115929.1, ZP_00013255.1

Moreover, an especially preferred hydroxylase is the hydroxylase from tomato (Accession Y14809) (nucleic acid: SEQ ID NO: 97; protein: SEQ ID NO. 98).
Examples of a β-cyclase gene are:
a nucleic acid encoding a β-cyclase from tomato (Accession X86452).(Nucleic acid: SEQ ID NO: 19, protein: SEQ ID NO: 20),

and β-cyclases of the following accession numbers:

S66350	lycopene beta-cyclase (EC 5.5.1.-) - tomato
CAA60119	lycopene synthase [Capsicum annuum]
S66349	lycopene beta-cyclase (EC 5.5.1.-) - common tobacco
CAA57386	lycopene cyclase [Nicotiana tabacum]
AAM21152	lycopene beta-cyclase [Citrus sinensis]
AAD38049	lycopene cyclase [Citrus x paradisi]

AAN86060 lycopene cyclase [Citrus unshiu]
AAF44700 lycopene beta-cyclase [Citrus sinensis]
AAK07430 lycopene beta-cyclase [Adonis palaestina]
AAG10429 beta cyclase [Tagetes erecta]
AAA81880 lycopene cyclase
AAB53337 Lycopene beta cyclase
AAL92175 beta-lycopene cyclase [Sandersonia aurantiaca]
CAA67331 lycopene cyclase [Narcissus pseudonarcissus]
AAM45381 beta cyclase [Tagetes erecta]
AAO18661 lycopene beta-cyclase [Zea mays]
AAG21133 chromoplast-specific lycopene beta-cyclase [Lycopersicon esculentum]
AAF18989 lycopene beta-cyclase [Daucus carota]
ZP_001140 hypothetical protein [Prochlorococcus marinus str. MIT9313]
ZP_001050 hypothetical protein [Prochlorococcus marinus subsp. pastoris str. CCMP1378]
ZP_001046 hypothetical protein [Prochlorococcus marinus subsp. pastoris str. CCMP1378]
ZP_001134 hypothetical protein [Prochlorococcus marinus str. MIT9313]
ZP_001150 hypothetical protein [Synechococcus sp. WH 8102]
AAF10377 lycopene cyclase [Deinococcus radiodurans]
BAA29250 393aa long hypothetical protein [Pyrococcus horikoshii]
BAC77673 lycopene beta-monocyclase [marine bacterium P99-3]
AAL01999 lycopene cyclase [Xanthobacter sp. Py2]
ZP_000190 hypothetical protein [Chloroflexus aurantiacus]
ZP_000941 hypothetical protein [Novosphingobium aromaticivorans]
AAF78200 lycopene cyclase [Bradyrhizobium sp. ORS278]
BAB79602 crtY [Pantoea agglomerans pv. milletiae]
CAA64855 lycopene cyclase [Streptomyces griseus]
AAA21262 dycopene cyclase [Pantoea agglomerans]
C37802 crtY protein - Erwinia uredovora
BAB79602 crtY [Pantoea agglomerans pv. milletiae]
AAA64980 lycopene cyclase [Pantoea agglomerans]
AAC44851 lycopene cyclase
BAA09593 Lycopene cyclase [Paracoccus sp. MBIC1143]
ZP_000941 hypothetical protein [Novosphingobium aromaticivorans]
CAB56061 lycopene beta-cyclase [Paracoccus marcusii]
BAA20275 lycopene cyclase [Erythrobacter longus]
ZP_000570 hypothetical protein [Thermobifida fusca]
ZP_000190 hypothetical protein [Chloroflexus aurantiacus]
AAK07430 lycopene beta-cyclase [Adonis palaestina]
CAA67331 lycopene cyclase [Narcissus pseudonarcissus]
AAB53337 Lycopene beta cyclase
BAC77673 lycopene beta-monocyclase [marine bacterium P99-3]

Furthermore, an especially preferred β-cyclase is the chromoplast-specific β-cyclase from tomato (AAG21133) (nucleic acid: SEQ ID No. 95; protein: SEQ ID No. 96)

Thus, in the transgenic plants which are preferred in accordance with the invention, at least one further hydroxylase gene and/or β-cyclase gene is present in this preferred embodiment in comparison with the wild type.

In this preferred embodiment, the genetically modified plant has, for example, at least one exogenous nucleic acid encoding a hydroxylase or at least two endogenous nucleic acids encoding a hydroxylase and/or at least one exogenous nucleic acid encoding a β-cyclase or at least two endogenous nucleic acids encoding a β-cyclase.

Hydroxylase genes which are preferably used in the above-described preferred embodiment are nucleic acids which encode proteins comprising the amino acid sequence SEQ ID NO: 18 or a sequence which is derived from this sequence by substitution, insertion or deletion of amino acids and which have at least 30%, by preference at least 50%, more preferably at least 70%, even more preferably at least 90%, most preferably at least 95% identity at the amino acid level with the sequence SEQ ID NO: 18 and which have the enzymatic property of a hydroxylase.

Further examples of hydroxylases and hydroxylase genes can be found readily, as described above, for example from various organisms whose genomic sequence is known, by homology comparisons of the amino acid sequences or of the corresponding backtranslated nucleic acid sequences from databases with SEQ ID NO: 18.

Further examples of hydroxylases and hydroxylase genes can furthermore be found readily in the manner known per se from various organisms whose genomic sequence is not known, by hybridization and PCR techniques as described above, for example starting from the sequence SEQ ID NO: 17.

In a further especially preferred embodiment, nucleic acids which encode proteins comprising the amino acid sequence of the hydroxylase of the sequence SEQ ID NO: 18 are introduced into organisms in order to increase the hydroxylase activity.

For example, suitable nucleic acid sequences can be obtained by backtranslation of the polypeptide sequence in accordance with

the genetic code.

Codons which are preferably used for this purpose are codons which are used frequently in accordance with the plant-specific codon usage. The codon usage can be determined readily with the aid of computer evaluations of other, known genes of the organisms in question.

In an especially preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 17 is introduced into the organism.

β-Cyclase genes which are preferably used in the above-described preferred embodiment are nucleic acids which encode proteins comprising the amino acid sequence SEQ ID NO: 20 or a sequence which is derived from this sequence by substitution, insertion or deletion of amino acids and which have at least 30%, by preference at least 50%, more preferably at least 70%, even more preferably at least 90%, most preferably at least 95% identity at the amino acid level with the sequence SEQ ID NO: 20 and which have the enzymatic property of a β-cyclase.

Further examples of β-cyclases and β-cyclase genes can be found readily, as described above, for example from various organisms whose genomic sequence is known, by homology comparisons of the amino acid sequences or of the corresponding backtranslated nucleic acid sequences from databases with SEQ ID NO: 20.

Further examples of β-cyclases and β-cyclase genes can furthermore be found readily in the manner known per se from various organisms whose genomic sequence is not known, by hybridization and PCR techniques, for example starting from the sequence SEQ ID NO: 19.

In a further especially preferred embodiment, nucleic acids which encode proteins comprising the amino acid sequence of the β-cyclase of the sequence SEQ ID NO: 20 are introduced into organisms in order to increase the β-cyclase activity.

For example, nucleic acid sequences can be obtained by backtranslation of the polypeptide sequence in accordance with the genetic code.

Codons which are preferably used for this purpose are codons which are frequently used in accordance with the plant-specific codon usage. The codon usage can be determined readily with the aid of computer evaluations of other, known genes of the

organisms in question.

In an especially preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 19 is introduced into the organism.

All of the abovementioned hydroxylase genes or β-cyclase genes can furthermore be generated in a known manner by chemical synthesis, starting with the nucleotide units, such as, for example, by fragment condensation of individual overlapping, complementary nucleic acid units of the double helix. Oligonucleotides can be synthesized chemically in a known manner for example by the phosphoamidite method (Voet, Voet, 2nd Edition, Wiley Press New York, pp. 896-897). The annealing of synthetic oligonucleotides and filling in of gaps by means of the Klenow fragment of the DNA polymerase and ligation reactions, and general cloning methods, are described in Sambrook et al. (1989), Molecular cloning: A laboratory manual, Cold Spring Harbor Laboratory Press.

In a further preferred embodiment of the method, the plants additionally show a reduced ε-cyclase activity in comparison with the wild type.

ε-Cyclase activity is understood as meaning the enzyme activity of an ε-cyclase.

An ε-cyclase is understood as meaning a protein with the enzymatic activity of converting a terminal, linear lycopene residue into an ε-ionone ring.

Thus, an ε-cyclase is understood as meaning in particular a protein with enzymatic activity of converting lycopene into δ-carotene.

Accordingly, ε-cyclase activity is understood as meaning the amount of lycopene converted, or the amount of δ-carotene formed, by the protein ε-cyclase in a certain period of time.

Thus, in the case of a reduced ε-cyclase activity in comparison with the wild type, the amount of lycopene converted, or the amount of δ-carotene formed, by the protein ε-cyclase is reduced within a certain period of time in comparison with the wild type.

A reduced ε-cyclase activity is preferably understood as meaning the partially or essentially complete prevention or blocking of the functionality of an ε-cyclase in a plant cell, plant or a part, tissue, organ, cell or seed derived therefrom, which is

based on different cell-biological mechanisms.

Reducing the ε-cyclase activity in plants in comparison with the wild type can be effected for example by reducing the amount of ε-cyclase protein, or the amount of ε-cyclase mRNA, in the plant. Accordingly, an ε-cyclase activity which is reduced in comparison with the wild type can be determined directly or via the determination of the amount of ε-cyclase protein or the amount of ε-cyclase mRNA of the plant according to the invention in comparison with the wild type.

A reduction of the ε-cyclase activity comprises a quantitative reduction of an ε-cyclase down to an essentially complete absence of ε-cyclase (i.e. lack of detectability of ε-cyclase activity, or lack of immunological detectability of ε-cyclase). Preferably, the ε-cyclase activity (or the amount of ε-cyclase protein or the amount of ε-cyclase mRNA) in the plant, especially preferably in flowers, is reduced by at least 5%, further preferably by at least 20%, further preferably by at least 50%, further preferably by 100%, in comparison with the wild type. In particular, "reduction" also means the complete absence of ε-cyclase activity (or of the ε-cyclase protein or the ε-cyclase mRNA).

The ε-cyclase activity in genetically modified plants according to the invention and in wild-type, or reference, plants is preferably determined under the following conditions:

The ε-cyclase activity may be determined *in vitro* by the method of Fraser and Sandmann (Biochem. Biophys. Res. Comm. 185(1) (1992) 9-15), when the following are added to a certain amount of plant extract: potassium phosphate to act as buffer (pH 7.6), lycopene to act as substrate, stromaprotein from Capsicum, NADP+, NADPH and ATP.

The ε-cyclase activity in genetically modified plants according to the invention and in wild-type, or reference, plants is especially preferably determined by the method of Bouvier, d'Harlingue and Camara (Molecular Analysis of carotenoid cyclase inhibition; Arch. Biochem. Biophys. 346(1) (1997) 53-64) under the following conditions:

The in-vitro assay is carried out in a volume of 0.25 ml. The mixture comprises 50 mM potassium phosphate (pH 7.6), different amounts of plant extract, 20 nM of lycopene, 0.25 mg of chromoplastidial stromaprotein from Capsicum, 0.2 mM NADP+, 0.2 mM NADPH and 1 mM ATP. NADP/NADPH and ATP are dissolved in 0.01 ml of ethanol together with 1 mg of Tween 80 immediately

prior to addition to the incubation medium. After a reaction time of 60 minutes at 30°C, the reaction is quenched by addition of chloroform/methanol (2:1). The reaction products which are extracted in chloroform are analyzed by means of HPLC.

An alternative assay in which radioactive substrate is used is described in Fraser and Sandmann (Biochem. Biophys. Res. Comm. 185(1) (1992) 9-15). A further analytical method is described in Beyer, Kröncke and Nievelstein (On the mechanism of the lycopene isomerase/cyclase reaction in Narcissus pseudonarcissus L. chromopast,; J. Biol. Chem. 266(26) (1991) 17072-17078).

Preferably, the ε-cyclase activity in plants is effected by at least one of the following methods:

a) introducing at least one double-stranded ε-cyclase ribonucleic acid sequence, also referred to as ε-cyclase dsRNA, hereinbelow or (an) expression cassette(s) which ensure(s) its expression. Comprised are those methods in which the ε-cyclase dsRNA is directed against an ε-cyclase gene (i.e. genomic DNA sequences, such as the promoter sequence) or an ε-cyclase transcript (i.e. mRNA sequences),

b) introducing at least one ε-cyclase antisense ribonucleic acid sequence, hereinbelow also referred to as ε-cyclase antisense RNA, or an expression cassette which ensures its expression. Comprised are those methods in which the ε-cyclase antisense RNA is directed against an ε-cyclase gene (i.e. genomic DNA sequences) or an ε-cyclase gene transcript (i.e. RNA sequences). Also comprised are α-anomeric nucleic acid sequences,

c) introducing at least one ε-cyclase antisense RNA in combination with a ribozyme or (an) expression cassette(s) which ensure(s) its expression,

d) introducing at least one ε-cyclase sense ribonucleic acid sequence, hereinbelow also referred to as ε-cyclase sense RNA, for inducing a cosuppression or an expression cassette which ensures its expression,

e) introducing at least one DNA- or protein-binding factor against an ε-cyclase gene, an ε-cyclase RNA or an ε-cyclase protein or an expression cassette which ensures its expression,

f) introducing at least one viral nucleic acid sequence which brings about the degradation of ε-cyclase RNA or an expression cassette which ensures its expression,

g) introducing at least one construct for generating a loss of function, such as, for example, the generation of stop

codons or a reading-frame shift, at an ε-cyclase gene, for example by generating an insertion, deletion, inversion or mutation in an ε-cyclase gene. Preferably, knock-out mutants can be generated by means of site-specific insertion into said ε-cyclase gene by means of homologous recombination or introduction of sequence-specific nucleases against ε-cyclase gene sequences.

The skilled worker is familiar with the fact that other methods may also be employed within the scope of the present invention for reducing an ε-cyclase or its activity or function. For example, the introduction of a dominant-negative variant of an ε-cyclase, or of an expression cassette which ensures its expression, may also be advantageous. Here, each and any of these methods may bring about a reduction of the amount of protein, the amount of mRNA and/or the activity of an ε-cyclase. A combined application is also feasible. Further methods are known to the skilled worker and may comprise the prevention or repression of the processing of ε-cyclase, of the transport of ε-cyclase or its mRNA, inhibition of ribosome attachment, inhibition of RNA splicing, induction of an ε-cyclase-RNA-degrading enzyme and/or inhibition of the elongation or termination of the translation.

The individual preferred variants may now be described by examples of embodiments:

a) introduction of a double-stranded ε-cyclase ribonucleic acid sequence (ε-cyclase dsRNA)

The method of regulating genes by means of double-stranded RNA ("double-stranded RNA interference"; dsRNAi) is known and described, for example, in Matzke MA et al. (2000) Plant Mol Biol 43:401-415; Fire A. et al (1998) Nature 391:806-811; WO 99/32619; WO 99/53050; WO 00/68374; WO 00/44914; WO 00/44895; WO 00/49035 or WO 00/63364. The processes and methods described in the abovementioned references are expressly referred to herewith.

In accordance with the invention, "double-stranded ribonucleic acid sequence" is understood as meaning one or more ribonucleic acid sequences which are capable theoretically, owing to complementary sequences, for example in accordance with Waston and Crick's base pair rules, and/or in real terms, for example on the basis of hybridization experiments, of forming double-stranded RNA structures in vitro and/or in vivo.

The skilled worker is aware of the fact that the formation of double-stranded RNA structures constitutes a state of equilibrium. Preferably, the ratio of double-stranded molecules to corresponding dissociated forms amounts to at least 1 to 10, preferably 1:1, especially preferably 5:1, most preferably 10:1.

A double-stranded ε-cyclase ribonucleic acid sequence, or else ε-cyclase dsRNA, is preferably understood as meaning an RNA molecule which has a region with double-stranded structure and comprises, in this region, a nucleic acid sequence which

a) is identical to at least a part of the plant's homologous ε-cyclase transcript and/or

b) is identical to at least a part of the plant's homologous ε-cyclase promoter sequence.

Thus, it is preferred, in the method according to the invention, to introduce, in order to reduce the ε-cyclase activity, an RNA into the plant, which RNA has a region with duplex structure and comprises, in this region, a nucleic acid sequence with

a) is identical to at least a part of the plant's homologous ε-cyclase transcript and/or

b) is identical to at least a part of the plant's homologous ε-cyclase promoter sequence.

The term "ε-cyclase transcript" is understood as meaning the transcribed part of an ε-cyclase gene which, in addition to the ε-cyclase-coding sequence, for example also comprises noncoding sequences such as, for example, UTRs.

An RNA which "is identical to at least a part of the plant's homologous ε-cyclase promoter sequence" preferably means that the RNA sequence is identical to at least a part of the theoretical transcript of the ε-cyclase promoter sequence, i.e. to the corresponding RNA sequence.

"A part" of the plant's homologous ε-cyclase transcript, or the plant's homologous ε-cyclase promoter sequence, is understood as meaning part-sequences which may reach from a few base pairs up to complete sequences of the transcript, or of the promoter sequence. The skilled worker can readily determine the optimal length of the part-sequences by routine experimentation.

As a rule, the length of the part-sequences amounts to at least 10 bases and not more than 2 kb, preferably at least 25 bases and not more than 1.5 kb, especially preferably at least 50 bases and not more than 600 bases, very especially preferably at least 100 bases and not more than 500, most preferably at least 200 bases or at least 300 bases and not more than 400 bases.

Preferably, the part-sequences are selected in such a way that as high a specificity as possible is achieved and that it is avoided to reduce activities of other enzymes whose reduction is not desired. Thus, it is advantageous to select, for the part-sequences of the ε-cyclase dsRNA, parts of the ε-cyclase transcripts and/or part-sequences of the ε-cyclase promoter sequences which are not found in other activities.

Thus, in an especially preferred embodiment, the ε-cyclase dsRNA comprises a sequence which is identical to a part of the plant's homologous ε-cyclase transcript and which comprises the 5'terminus or the 3'terminus of the plant's homologous nucleic acid encoding an ε-cyclase. Untranslated regions 5' or 3' of the transcript are especially suitable for generating selective double-stranded structures.

The invention furthermore relates to double-stranded RNA molecules (dsRNA molecules) which, when introduced into a plant organism (or a cell, tissue, organ or propagation material derived therefrom), bring about the reduction of an ε-cyclase.

In this context, a double-stranded RNA molecule for reducing the expression of an ε-cyclase (ε-cyclase dsRNA) preferably comprises

a) a sense RNA strand comprising at least one ribonucleotide sequence which is essentially identical to at least a part of a sense RNA ε-cyclase transcript, and

b) an antisense RNA strand which is essentially, preferably fully, complementary to the RNA sense strand of a).

To transform the plant with an ε-cyclase dsRNA, it is preferred to use a nucleic acid construct which is introduced into the plant and which is transcribed in the plant into the ε-cyclase dsRNA.

Thus, the present invention also relates to a nucleic acid construct which can be transcribed into

a) a sense RNA strand comprising at least one ribonucleotide sequence which is essentially identical to at least a part of

the sense RNA ε-cyclase transcript, and

b) an antisense RNA strand which is essentially, preferably fully, complementary to the RNA sense strand of a).

These nucleic acid constructs are hereinbelow also referred to as expression cassettes or expression vectors.

As regards the dsRNA molecules, ε-cyclase nucleic acid sequence, or the corresponding transcript, is preferably understood as meaning the sequence in accordance with SEQ ID NO: 38 or a part of the same.

"Essentially identical" means that the dsRNA sequence may also comprise insertions, deletions and individual point mutations in comparison with the ε-cyclase target sequence while still bringing about an efficient reduction of the expression. Preferably, the homology amounts to at least 75%, preferably at least 80%, very especially preferably at least 90%, most preferably 100%, between the sense strand of an inhibitory dsRNA and at least a part of the sense RNA transcript of an ε-cyclase gene, or between the antisense strand, the complementary strand of an ε-cyclase gene.

100% sequence identity between dsRNA and an ε-cyclase gene transcript is not necessarily required in order to bring about an efficient reduction of the ε-cyclase expression. Accordingly, there is the advantage that the method is tolerant to sequence deviations as can be present as a result of genetic mutations, polymorphisms or evolutionary divergences. Thus, for example, using the dsRNA which has been generated starting from the ε-cyclase sequence of the one organism, it is possible to suppress the ε-cyclase expression in another organism. To this end, the dsRNA preferably comprises sequence regions of ε-cyclase gene transcripts which correspond to conserved regions. Said conserved regions can be deduced readily from sequence comparisons.

As an alternative, an "essentially identical" dsRNA can also be defined as a nucleic acid sequence which is capable of hybridizing with a part of an ε-cyclase gene transcript (for example in 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA at 50°C or 70°C for 12 to 16 h).

"Essentially complementary" means that the antisense RNA strand may also show insertions, deletions and individual point mutations in comparison with the complement of the sense RNA strand. Preferably, the homology amounts to at least 80%, preferably at least 90%, very especially preferably at least 95%,

most preferably 100%, between the antisense RNA strand and the complement of the sense RNA strand.

In a further embodiment, the ε-cyclase dsRNA comprises

a) a sense RNA strand comprising at least one ribonucleotide sequence which is essentially identical to at least a part of the sense RNA transcript of the promoter region of an ε-cyclase gene, and

b) an antisense RNA strand which is essentially, preferably fully, complementary to the RNA sense strand of a).

The corresponding nucleic acid construct which is preferably used for the transformation of the plants comprises

a) a sense DNA strand which is essentially identical to at least a part of the promoter region of an ε-cyclase gene, and

b) an antisense DNA strand which is essentially, preferably fully, complementary to the DNA sense strand of a).

Preferably, the promoter region of an ε-cyclase is understood as meaning a sequence as shown in SEQ ID NO: 47 or a part of the same.

To generate the ε-cyclase dsRNA sequences for reducing the ε-cyclase activity, the following part-sequences are especially preferably used, in particular for *Tagetes erecta*:

SEQ ID NO: 40: Sense fragment of the 5'-terminal region of the ε-cyclase

SEQ ID NO: 41: Antisense fragment of the 5'-terminal region of the ε-cyclase

SEQ ID NO: 42: Sense fragment of the 3'-terminal region of the ε-cyclase

SEQ ID NO: 43: Antisense fragment of the 3'-terminal region of the ε-cyclase

SEQ ID NO: 47: Sense fragment of the ε-cyclase promoter

SEQ ID NO: 48: Antisense fragment of the ε-cyclase promoter

The dsRNA can consist of one or more strands of polyribonucleotides. To achieve the same purpose, it is, naturally, also possible to introduce, into the cell or the organism, several individual dsRNA molecules, each of which comprises one of the above-defined ribonucleotide sequence segments.

The double-stranded dsRNA structure can be formed starting from two complementary separate RNA strands or – preferably – starting from an individual autocomplementary RNA strand. In this case, sense RNA strand and antisense RNA strand are preferably covalently linked with one another in the form of an inverted repeat.

As described for example in WO 99/53050, the dsRNA may also comprise a hairpin structure by sense and antisense strand being linked by a linking sequence (linker; for example an intron). The autocomplementary dsRNA structures are preferred since they merely require the expression of one RNA sequence and always comprise the complementary RNA strands in an equimolar ratio. The linking sequence is preferably an intron (for example an intron of the potato ST-LS1 gene; Vancanneyt GF et al. (1990) Mol Gen Genet 220(2):245-250).

The nucleic acid sequence encoding a dsRNA may comprise further elements such as, for example, transcription termination signals or polyadenylation signals.

However, if the dsRNA is directed against the promoter sequence of an ε-cyclase, it preferably comprises no transcription termination signals or polyadenylation signals. This makes possible a retention of the dsRNA in the nucleus of the cell and prevents spreading of the dsRNA in all of the plant.

If the two strands of the dsRNA are to be combined in one cell or plant, this can be effected for example in the following manner:

a) transformation of the cell or plant with a vector which comprises both expression cassettes,

b) cotransformation of the cell or plant with two vectors, one comprising the expression cassettes with the sense strand, while the other comprises the expression cassettes with the antisense strand.

c) Crossing of two individual plant lines, one comprising the expression cassettes with the sense strand while the other

comprises the expression cassettes with the antisense strand.

The formation of the RNA double-stranded can be initiated either outside the cell or within the same.

The dsRNA can be synthesized either in vivo or in vitro. To this end, a DNA sequence encoding a dsRNA can be introduced into an expression cassette under the control of at least one genetic control element (such as, for example, a promoter). Polyadenylation is not required, nor do elements for initiating a translation have to be present. The expression cassette for the ε-cyclase dsRNA is preferably present on the transformation construct or the transformation vector.

In an especially preferred embodiment, the expression of the dsRNA takes place starting from an expression construct under the functional control of a flower-specific promoter, especially preferably under the control of the promoter described by SEQ ID NO: 28 or a functional equivalent part thereof.

The expression cassettes encoding the antisense and/or the sense strand of an ε-cyclase dsRNA or the autocomplementary strand of the dsRNA are preferably inserted into a transformation vector and introduced into the plant cell using the methods described hereinbelow. A stable insertion into the genome is advantageous for the method according to the invention.

The dsRNA can be introduced in an amount which makes possible at least one copy per cell. If appropriate, larger amounts (for example at least 5, 10, 100, 500 or 1000 copies per cell) may bring about a more efficient reduction.

b) Introducing an antisense ribonucleic acid sequence of an ε-cyclase (ε-cyclase antisense RNA)

Methods for reducing a certain protein by means of the antisense technology have been described on many occasiosn, including in plants (Sheehy et al. (1988) Proc Natl Acad Sci USA 85: 8805-8809; US 4,801,340; Mol JN et al. (1990) FEBS Lett 268(2):427-430). The antisense nucleic acid molecule hybridizes with, or binds to, cellular mRNA and/or genomic DNA encoding the ε-cyclase to be reduced. The transcription and/or translation of the ε-cyclase is thereby suppressed. The hybridization can be brought about in the traditional manner via the formation of a stable double-stranded or – in the case of genomic DNA – by binding the antisense nucleic acid molecule with the double-stranded of the genomic DNA by specific interaction in the

large groove of the DNA helix.

An ε-cyclase antisense RNA can be derived using the nucleic acid sequence encoding this ε-cyclase, for example the nucleic acid sequence as shown in SEQ ID NO: 38, following the base-pairing rules of Watson and Crick. The ε-cyclase antisense RNA can be complementary to all of the transcribed mRNA of the ε-cyclase, it may be limited to the coding region, or else it may consist of only one oligonucleotide which is complementary to a part of the coding or noncoding sequence of the mRNA. Thnus, the oligonucleotide can, for example, be complementary to the region which comprises the translation start for the ε-cyclase. The ε-cyclase antisense RNA can have a length of, for example, 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides, but can also be longer and comprise at least 100, 200, 500, 1000, 2000 or 5000 nucleotides. For the purposes of the present invention, ε-cyclase antisense RNAs are preferably expressed recombinantly in the target cell.

The invention furthermore relates to transgenic expression cassettes comprising a nucleic acid sequence encoding at least a part of an ε-cyclase, where said nucleic acid sequence is functionally linked in antisense orientation with a promoter which is functional in plant organisms. In an especially preferred embodiment, the expression of the antisense RNA takes place starting from an expression construct under the functional control of a flower-specific promoter, especially preferably under the control of the promoter described by SEQ ID NO: 28 or a functional equivalent part thereof.

Said expression cassettes can be part of a transformation construct or transformation vector, or else be introduced in context with a cotransformation.

In a further preferred embodiment, the expression of an ε-cyclase can be inhibited by nucleotide sequences which are complementary to the regulatory region of an ε-cyclase gene (for example an ε-cyclase promoter and/or enhancer) and which form triple-helical structures with the DNA double helix therein, so that the transcription of the ε-cyclase gene is reduced. Such methods have been described (Helene C (1991) Anticancer Drug Res 6(6):569-84; Helene C et al. (1992) Ann NY Acad Sci 660:27-36; Maher LJ (1992) Bioassays 14(12):807- 815).

In a further embodiment, the ε-cyclase antisense RNA can be an α-anomeric nucleic acid. Such α-anomeric nucleic acid molecules form specific double-stranded hybrids with complementary RNA in

which – in contrast to the conventional β-nucleic acids – the two strands run parallel with one another (Gautier C et al. (1987) Nucleic Acids Res 15:6625-6641).

c) Introducing an ε-cyclase antisense RNA in combination with a ribozyme

The above-described antisense strategy can advantageously be coupled with a ribozyme method. Catalytic RNA molecules, or ribozymes, can be adapted to suit any target RNA and cleave the phosphodiester backbone at specific positions, thus functionally deactivating the target RNA (Tanner NK (1999) FEMS Microbiol Rev 23(3):257-275). The ribozyme itself is not modified thereby, but is capable of cleaving further target RNA molecules analogously, thus assuming the characteristics of an enzyme. The incorporation of ribozyme sequences into antisense RNAs imparts this enzyme-like, RNA-cleaving characteristic to precisely these antisense RNAs and thus increases their efficiency in inactivation of the target RNA. The generation and use of such ribozyme antisense RNA molecules is described (inter alia in Haselhoff et al. (1988) Nature 334: 585-591); Haselhoff und Gerlach (1988) Nature 334:585-591; Steinecke P et al. (1992) EMBO J 11(4):1525-1530; de Feyter R et al. (1996) Mol Gen Genet. 250(3):329-338).

In this manner, it is possible to use ribozymes (for example hammerhead ribozymes; Haselhoff and Gerlach (1988) Nature 334:585-591) in order to catalytically cleave the mRNA of an ε-cyclase to be reduced and thus preventing translation. The ribozyme technology can increase the efficiency of an antisense strategy. Methods for the expression of ribozymes for reducing certain proteins are described in (EP 0 291 533, EP 0 321 201, EP 0 360 257). Ribozyme expression in plant cells is likewise described (Steinecke P et al. (1992) EMBO J 11(4):1525-1530; de Feyter R et al. (1996) Mol Gen Genet. 250(3):329-338). Suitable target sequences and ribozymes can be determined as described, for example, in "Steinecke P, Ribozymes, Methods in Cell Biology 50, Galbraith et al. eds, Academic Press, Inc. (1995), pp. 449-460", by calculating the secondary structures of ribozyme RNA and target RNA, and by their interaction (Bayley CC et al. (1992) Plant Mol Biol. 18(2):353-361; Lloyd AM and Davis RW et al. (1994) Mol Gen Genet. 242(6):653-657). For example, it is possible to construct derivatives of the Tetrahymena L-19 IVS RNA which have regions which are complementary to the mRNA of the ε-cyclase to be suppressed (see also US 4,987,071 and US 5,116,742). As an alternative, such ribozymes can also be identified from a library of various ribozymes via a selection

process (Bartel D and Szostak JW (1993) Science 261:1411-1418).

d) Introducing a sense ribonucleic acid sequence of an ε-cyclase (ε-cyclase sense RNA) for inducing cosuppression

The expression of an ε-cyclase ribonucleic acid sequence (or a part thereof) in sense orientation can lead to a cosuppression of the corresponding ε-cyclase gene. The expression of sense RNA with homology to an endogenous ε-cyclase gene can reduce or eliminate the expression of the same, in a similar manner as has been described for antisense approaches (Jorgensen et al. (1996) Plant Mol Biol 31(5):957-973; Goring et al. (1991) Proc Natl Acad Sci USA 88:1770-1774; Smith et al. (1990) Mol Gen Genet 224:447-481; Napoli et al. (1990) Plant Cell 2:279-289; Van der Krol et al. (1990) Plant Cell 2:291-99). In this context, the construct introduced can represent all or only some of the homologous gene to be reduced. The possibility of translation is not required. The application of this technology to plants is described (for example Napoli et al. (1990) Plant Cell 2:279-289; in US 5,034,323.

Preferably, cosuppression is realized using a sequence which is essentially identical to at least a part of the nucleic acid sequence encoding an ε-cyclase, for example the nucleic acid sequence as shown in SEQ ID NO: 38.

Preferably, the ε-cyclase sense RNA is selected in such a way that a translation of the ε-cyclase , or a part thereof, is not possible. To this end, it is possible for example to choose the 5'-untranslated or 3'-untranslated region, or else to delete or mutate the ATG start codon.

e) Introducing DNA- or protein-binding factors against ε-cyclase genes, ε-cyclase RNAs or ε-cyclase proteins

A reduction of an ε-cyclase expression is also possible using specific DNA-binding factors, for example factors of the zinc finger transcription factor type. These factors attach to the genomic sequence of the endogenous target gene, preferably in the regulatory regions, and bring about a reduction of the expression. Suitable methods for the generation of such factors are described (Dreier B et al. (2001) J Biol Chem 276(31):29466-78; Dreier B et al. (2000) J Mol Biol 303(4):489-502; Beerli RR et al. (2000) Proc Natl Acad Sci USA 97 (4):1495-1500; Beerli RR et al. (2000) J Biol Chem 275(42):32617-32627; Segal DJ and Barbas CF 3rd. (2000) Curr Opin Chem Biol 4(1):34-39; Kang JS and Kim JS (2000) J Biol Chem

275(12):8742-8748; Beerli RR et al. (1998) Proc Natl Acad Sci USA 95(25):14628-14633; Kim JS et al. (1997) Proc Natl Acad Sci USA 94(8):3616-3620; Klug A (1999) J Mol Biol 293(2):215-218; Tsai SY et al. (1998) Adv Drug Deliv Rev 30(1-3):23-31; Mapp AK et al. (2000) Proc Natl Acad Sci USA 97(8):3930-3935; Sharrocks AD et al. (1997) Int J Biochem Cell Biol 29(12):1371-1387; Zhang L et al. (2000) J Biol Chem 275(43):33850-33860).

These factors can be selected using any portion of an ε-cyclase gene. This segment is preferably in the promoter region. To suppress a gene, however, it may also be in the region of the coding exons or introns.

Furthermore, it is possible to introduce, into a cell, factors which inhibit the ε-cyclase itself. These protein-binding factors can be for example aptamers (Famulok M and Mayer G (1999) Curr Top Microbiol Immunol 243:123-36) or antibodies, or antibody fragments, or single-chain antibodies. The preparation of these factors is described (Owen M et al. (1992) Biotechnology (N Y) 10(7):790-794; Franken E et al. (1997) Curr Opin Biotechnol 8(4):411-416; Whitelam (1996) Trend Plant Sci 1:286-272).

f) Introducing viral nucleic acid sequences and expression constructs which bring about the degradation of ε-cyclase RNA

The expression of ε-cyclase can also be carried out effectively by inducing the specific ε-cyclase RNA degradation by the plant with the aid of a viral expression system (amplicon; Angell SM et al. (1999) Plant J 20(3):357-362). These systems – also referred to as VIGS (viral-induced gene silencing) – introduce, into the plant, nucleic acid sequences with homology to the transcript of an ε-cyclase to be reduced, using viral vectors. Then, transcription is switched off, probably mediated by plant defence mechanisms against viruses. Such techniques and methods are described (Ratcliff F et al. (2001) Plant J 25(2):237-45; Fagard M und Vaucheret H (2000) Plant Mol Biol 43(2-3):285-93; Anandalakshmi R et al. (1998) Proc Natl Acad Sci USA 95(22):13079-84; Ruiz MT (1998) Plant Cell 10(6):937-46).

Preferably, the VIGS-induced reduction is carried out using a sequence which is esentially identical to at least a part of the nucleic acid sequence encoding an ε-cyclase, for example the nucleic acid sequence as shown in SEQ ID NO: 38.

g) Introducing constructs for generating a loss of function, or reduced function, on ε-cyclase genes

The skilled worker is familiar with a large number of methods for modifying genomic sequences in a site-specific manner. These include, in particular, methods such as the generation of knock-out mutants with the aid of site-specific homologous recombination, for example by generating stop codons, reading-frame shifts and the like (Hohn B and Puchta H (1999) Proc Natl Acad Sci USA 96:8321-8323) or the site-specific deletion or inversion of sequences by means of, for example, sequence-specific recombinases or nucleases (see hereinbelow).

The reduction of the amount, function and/or activity of the ε-cyclase can also be effected by a site-specific insertion of nucleic acid sequences (for example of the nucleic acid sequence to be inserted for the purposes of the method according to the invention) into the sequence encoding an ε-cyclase (for example by means of intermolecular homologous recombination). A DNA construct which is preferably used for the purposes of this embodiment is a construct which comprises at least a part of the sequence of an ε-cyclase gene or adjacent sequences, and is thus capable of undergoing site-specific recombination with them in the target cell, so that a deletion, addition or substitution of at least one nucleotide modifies the ε-cyclase gene in such a manner that the functionality of the ε-cyclase gene is reduced or completely eliminated. The modification may also affect the regulatory elements (for example the promoter) of the ε-cyclase gene so that the coding sequence remains unmodified, but expression (transcription and/or translation) does not take place and is reduced. In the case of conventional homologous recombination, the sequence to be inserted is flanked at its 5'- and/or 3'-terminus by further nucleic acid sequences (A' and B', respectively) which have sufficient length and sufficient homology with corresponding sequences of the ε-cyclase gene (A and B, respectively) for allowing homologous recombination. The length is, as a rule, in the range of from several hundred bases up to several kilobases (Thomas KR and Capecchi MR (1987) Cell 51:503; Strepp et al. (1998) Proc Natl Acad Sci USA 95(8):4368-4373). To carry out the homologous recombination, the plant cell is transformed with the recombination construct using the methods described hereinbelow, and clones which have successfully undergone recombination are selected based on the now inactivated ε-cyclase.

In a further preferred embodiment, the recombination efficiency is increased by being combined with methods which promote homologous recombination. Such methods are described and comprise, for example, the expression of proteins such a RecA or the treatment with PARP inhibitors. It has been demonstrated that

intrachromosomal homologous recombination in tobacco plants can be increaed by using PARP inhibitors (Puchta H et al. (1995) Plant J 7:203-210). By using these inhibitors, the homologous recombination rate in the recombination constructs after induction of the sequence-specific DNA double-strand break, and thus the efficiency of the deletion of the transgene sequences, can be increased further. It is possible to use various PARP inhibitors. Preferably comprised are inhibitors such as 3-aminobenzamide, 8-hydroxy-2-methylquinazolin-4-one (NU1025), 1,11b-dihydro-[2H]benzopyrano[4,3,2-de]isoquinolin-3-one (GPI 6150), 5-aminoisoquinolinone, 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2H)-isoquinolinone, or the substances described in WO 00/26192, WO 00/29384, WO 00/32579, WO 00/64878, WO 00/68206, WO 00/67734, WO 01/23386 and WO 01/23390.

Further suitable methods are the introduction of nonsense mutations into endogenous marker protein genes, for example by means of introducing RNA/DNA oligonucleotides into the plant (Zhu et al. (2000) Nat Biotechnol 18(5):555-558), or the generation of knock-out mutants with the aid of, for example, T-DNA mutagenesis (Koncz et al., Plant Mol. Biol. 1992, 20(5):963-976). Point mutations can also be generated by means of DNA/RNA hybrids, which are also known as "chimeraplasty" (Cole-Strauss et al. (1999) Nucl Acids Res 27(5):1323-1330; Kmiec (1999) Gene therapy American Scientist 87(3):240-247).

The methods dsRNAi, cosuppression by means of sense RNA and VIGS (virus-induced gene silencing) are also referred to as post-transcriptional gene silencing (PTGS) or transcritional gene silencing (TGS). PTGS/TGS methods are particularly advantageous since the requirements of the homology between the marker protein gene to be reduced and the transgenically expressed sense or dsRNA nucleic acid sequence are lower than, for example, in a traditional antisense approach. Thus, it is possible, using the marker protein nucleic acid sequences from one species, to reduce the expression of homologous marker protein proteins in other species effectively, without an isolation and structure elucidation of the marker protein homologues therein being mandatory. This considerably simplifies the work involved.

In an especially preferred embodiment of the method according to the invention, the ε-cyclase activity is reduced in comparison with the wild type by:

a) introducing, into plants, at least one double-stranded ε-cyclase ribonucleic acid sequence or (an) expression cassette(s) which ensure(s) its expression, and/or

b) introducing, into plants, at least one ε-cyclase antisense ribonucleic acid sequence or an expression cassette which ensures its expression.

In a very especially preferred embodiment, the reduction of the ε-cyclase activity in comparison with the wild type is effected by introducing, into plants, at least one double-stranded ε-cyclase ribonucleic acid sequence or (an) expression cassette(s) which ensure(s) its expression.

In a preferred embodiment, genetically modified plants are used which have the lowest expression rate of an ε-cyclase in flowers.

This is preferably achieved by the reduction of the ε-cyclase activity being effected in a flower-specific manner, especially preferably in a petal-specific manner.

In the above-described especially preferred embodiment, this is achieved by the transcription of the ε-cyclase dsRNA sequences being under the control of a flower-specific promoter, or even more preferably under the control of a petal-specific promoter.

In a further preferred embodiment, plants are cultured which, in comparison with the wild type, additionally show an increased activity selected from the group consisting of HMG-CoA reductase activity, (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity, 1-deoxy-D-xylose-5-phosphate synthase activity, 1-deoxy-D-xylose-5-phosphate reductoisomerase activity, isopentenyl-diphosphate Δ-isomerase activity, geranyl-diphosphate synthase activity, farnesyl-diphosphate synthase activity, geranylgeranyl-diphosphate synthase activity, phytoene synthase activity, phytoene desaturase activity, zeta-carotene desaturase activity, crtISO activity, FtsZ activity and MinD activity.

HMG-CoA reductase activity is understood as meaning the enzyme activity of an HMG-CoA reductase (3-hydroxy-3-methylglutaryl-coenzyme A reductase).

An HMG-CoA reductase is understood as meaning a protein with the enzymatic activity of converting 3-hydroxy-3-methylglutaryl-coenzyme A into mevalonate.

Accordingly, HMG-CoA reductase activity is understood as meaning the amount of 3-hydroxy-3-methylglutaryl-coenzyme A converted, or

the amount of mevalonate formed, by the protein HMG-CoA reductase within a certain period of time.

Thus, in the case of an increased HMG-CoA reductase activity in comparison with the wild-type, the amount of 3-hydroxy-3-methyl-glutaryl-coenzyme A converted, or the amount of mevalonate formed, by the protein HMG-CoA reductase within a certain period of time, is increased in comparison with the wild type.

By preference, this increase of HMG-CoA reductase activity amounts to at least 5%, further preferably to at least 20%, further preferably to at least 50%, further preferably to at least 100%, more preferably to at least 300%, even more preferably to at least 500%, in particular to at least 600%, of the HMG-CoA reductase activity of the wild type. HMG-CoA reductase activity is understood as meaning the enzyme activity of an HMG-CoA reductase.

HMG-CoA reductase activity in genetically modified plants according to the invention and in wild-type, or reference, plants is preferably determined under the following conditions:

Frozen plant material is homogenized by thoroughly crushing in liquid nitrogen and extracted with an extraction buffer in a ratio of from 1:1 to 1:20. The ratio in question depends on the enzyme activities in the plant material available, so that a determination and quantification of the enzyme activities within the linear measurement range are possible. Typically, the extraction buffer can consist of 50 mM HEPES-KOH (pH 7.4), 10 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.1% (v/v) Triton X-100, 2 mM ε-aminocaproic acid, 10% glycerol, 5 mM $KHCO_3$. 2 mM DTT and 0.5 mM PMSF are added shortly before the extraction.

The activity of the HMG-CoA reductase can be measured as described in published descriptions (for example Schaller, Grausem, Benveniste, Chye, Tan, Song and Chua, Plant Physiol. 109 (1995), 761-770; Chappell, Wolf, Proulx, Cuellar and Saunders, Plant Physiol. 109 (1995) 1337-1343). Plant tissue can be homogenized and extracted in cold buffer (100 mM potassium phosphate (pH 7.0), 4 mM $MgCl_2$, 5 mM DTT). The homogenate is centrifuged for 15 minutes at 10 000 g and 4°C. Thereafter, the supernatant is recentrifuged for 45-60 minutes at 100 000 g. The activity of the HMG-CoA reductase is determined in the supernatant and in the pellet of the microsomal fraction (after resuspending in 100 mM potassium phosphate (pH 7.0) and 50 mM DTT). Aliquots of the solution and of the suspension (the protein content of the suspension corresponds to approximately 1-10 µg)

are incubated for 15-60 minutes at 30°C in 100 mM potassium phosphate buffer (pH 7.0) with 3 mM NADPH and 20 µM (^{14}C)HMG-CoA (58 µCi/µM), ideally in a volume of 26 µl. The reaction is quenched by addition of 5 µl of mevalonate lactone (1 mg/ml) and 6 N HCl. After the addition, the mixture is incubated for 15 minutes at room temperature. The amount of (^{14}C)-mevalonate formed during the reaction is determined by adding 125 µl of a saturated potassium phosphate solution (pH 6.0) and 300 µl of ethyl acetate to the reaction mixture. The mixture is mixed thoroughly and centrifuged. The radioactivity can be determined by means of scintillation measurement.

(E)-4-Hydroxy-3-methylbut-2-enyl-diphosphate reductase activity, also referred to as lytB or IspH, is understood as meaning the enzyme activity of an (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase.

An (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase is understood as meaning a protein with the enzymatic activity of converting (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate into isopentenyl diphosphate and dimethylallyl diphosphate.

Accordingly, (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity is understood as meaning the amount of (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate converted, or the amount of isopentenyl diphosphate and/or dimethylallyl diphosphate formed, by the protein (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase within a certain period of time.

Thus, in the case of an increased (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity in comparison with the wild type, the amount of (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate converted, or the amount of isopentenyl diphosphate and/or dimethylallyl diphosphate formed, by the protein (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase within a certain period of time, is increased in comparison with the wild type.

By preference, this increase of (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity amounts to at least 5%, further preferably to at least 20%, further preferably to at least 50%, further preferably to at least 100%, more preferably to at least 300%, even more preferably to at least 500%, in particular to at least 600%, of the (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity of the wild type.

The (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity in genetically modified plants according to the invention and in wild-type, or reference, plants is preferably determined under the following conditions:

Frozen plant material is homogenized by thoroughly crushing in liquid nitrogen and extracted with an extraction buffer in a ratio of from 1:1 to 1:20. The ratio in question depends on the enzyme activities in the plant material available, so that a determination and quantification of the enzyme activities within the linear measurement range are possible. Typically, the extraction buffer can consist of 50 mM HEPES-KOH (pH 7.4), 10 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.1% (v/v) Triton X-100, 2 mM ε-aminocaproic acid, 10% glycerol, 5 mM $KHCO_3$. 2 mM DTT and 0.5 mM PMSF are added shortly before the extraction.

The (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity can be determined via an immunological detection. The production of specific antibodies has been described by Rohdich and coworkers (Rohdich, Hecht, Gärtner, Adam, Krieger, Amslinger, Arigoni, Bacher and Eisenreich: Studies on the nonmevalonate terpene biosynthetic pathway: metabolic role of IspH (LytB) protein, Natl. Acad. Natl. Sci. USA 99 (2002), 1158-1163). To determine the catalytic activity, Altincicek and coworkers (Altincicek, Duin, Reichenberg, Hedderich, Kollas, Hintz, Wagner, Wiesner, Beck and Jomaa: LytB protein catalyzes the terminal step of the 2-C-methyl-D-erythritol-4-phosphate pathway of isoprenoid biosynthesis; FEBS Letters 532 (2002), 437-440) describe an *in vitro* system which monitors the reduction of (E)-4-hydroxy-3-methyl-but-2-enyl diphosphate to isopentenyl diphosphate and dimethylallyl diphosphate.

1-Deoxy-D-xylose-5-phosphate synthase activity is understood as meaning the enzyme activity of a 1-deoxy-D-xylose-5-phosphate synthase.

A 1-deoxy-D-xylose-5-phosphate synthase is understood as meaning a protein with the enzymatic activity of converting hydroxyethyl-ThPP and glycerinaldehyde-3-phosphate into 1-deoxy-D-xylose-5-phosphate.

Accordingly, 1-deoxy-D-xylose-5-phosphate synthase activity is understood as meaning the amount of hydroxyethyl-ThPP and/or glycerinaldehyde-3-phosphate converted, or the amount of 1-deoxy-D-xylose-5-phosphate formed, by the protein

1-deoxy-D-xylose-5-phosphate synthase within a certain period of time.

Thus, in the case of an increased 1-deoxy-D-xylose-5-phosphate synthase activity in comparison with the wild type, the amount of hydroxyethyl-ThPP and/or glycerinaldehyde-3-phosphate converted, or the amount of 1-deoxy-D-xylose-5-phosphate formed, by the protein 1-deoxy-D-xylose-5-phosphate synthase within a certain period of time is increased in comparison with the wild type.

By preference, this increase of 1-deoxy-D-xylose-5-phosphate synthase activity amounts to at least 5%, further preferably to at least 20%, further preferably to at least 50%, further preferably to at least 100%, more preferably to at least 300%, even more preferably to at least 500%, in particular to at least 600%, of the 1-deoxy-D-xylose-5-phosphate synthase activity of the wild type.

The 1-deoxy-D-xylose-5-phosphate synthase activity in genetically modified plants according to the invention and in wild-type, or reference, plants is preferably determined under the following conditions:

Frozen plant material is homogenized by thoroughly crushing in liquid nitrogen and extracted with an extraction buffer in a ratio of from 1:1 to 1:20. The ratio in question depends on the enzyme activities in the plant material available, so that a determination and quantification of the enzyme activities within the linear measurement range are possible. Typically, the extraction buffer can consist of 50 mM HEPES-KOH (pH 7.4), 10 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.1% (v/v) Triton X-100, 2 mM ε-aminocaproic acid, 10% glycerol, 5 mM $KHCO_3$. 2 mM DTT and 0.5 mM PMSF are added shortly before the extraction.

The reaction solution (50-200 µl) for the determination of the D-1-deoxyxylulose-5-phosphate synthase activity (DXS) consists of 100 mM Tris-HCl (pH 8.0), 3 mM $MgCl_2$, 3 mM $MnCl_2$, 3 mM ATP, 1 mM thiamine diphosphate, 0.1% Tween-60, 1 mM potassium fluoride, 30 µM (2-^{14}C)-pyruvate (0.5 µCi), 0.6 mM DL-glyerinaldehyde-3-phosphate. The plant extract is incubated in the reaction solution for 1 to 2 hours at 37°C. Thereafter, the reaction is quenched by heating for 3 minutes at 80°C. After centrifugation at 13 000 revolutions/minute for 5 minutes, the supernatant is evaporated, the residue is resuspended in 50 µl of methanol, applied to a TLC plate for thin-layer chromatography (Silica-Gel 60, Merck, Darmstadt) and separated in N-propyl alcohol/ethyl acetate/water (6:1:3; v/v/v). During this process, radiolabeled

D-1-deoxyxylulose-5-phosphate (or D-1-deoxyxylulose) is separated from (2-^{14}C)-pyruvate. The quantitative determination is carried out by means of scintillation counter. The method was described in Harker and Bramley (FEBS Letters 448 (1999) 115-119). As an alternative, a fluorimetric assay for determining the DXS synthesis activity has been described by Querol and coworkers (Analytical Biochemistry 296 (2001) 101-105).

1-Deoxy-D-xylose-5-phosphate reductoisomerase activity is understood as meaning the enzyme activity of a 1-deoxy-D-xylose-5-phosphate reductoisomerase, also called 1-deoxy-D-xylulose-5-phosphate reductoisomerase.

A 1-deoxy-D-xylose-5-phosphate reductoisomerase is understood as meaning a protein with the enzymatic activity of converting 1-deoxy-D-xylose-5-phosphate into β-carotene.

Accordingly, 1-deoxy-D-xylose-5-phosphate reductoisomerase activity is understood as meaning the amount of 1-deoxy-D-xylose-5-phosphate converted, or the amount of isopentenyl diphosphate formed, by the protein 1-deoxy-D-xylose-5-phosphate reductoisomerase within a certain period of time.

Thus, in the case of an increased 1-deoxy-D-xylose-5-phosphate reductoisomerase activity in comparison with the wild type, the amount of 1-deoxy-D-xylose-5-phosphate converted, or the amount of isopentenyl diphosphate formed, by the protein 1-deoxy-D-xylose-5-phosphate reductoisomerase within a certain period of time is increased in comparison with the wild type.

By preference, this increase of 1-deoxy-D-xylose-5-phosphate reductoisomerase activity amounts to at least 5%, further preferably to at least 20%, further preferably to at least 50%, further preferably to at least 100%, more preferably to at least 300%, even more preferably to at least 500%, in particular to at least 600%, of the 1-deoxy-D-xylose-5-phosphate reductoisomerase activity of the wild type.

The 1-deoxy-D-xylose-5-phosphate reductoisomerase activity in genetically modified plants according to the invention and in wild-type, or reference, plants is preferably determined under the following conditions:

Frozen plant material is homogenized by thoroughly crushing in liquid nitrogen and extracted with an extraction buffer in a ratio of from 1:1 to 1:20. The ratio in question depends on the

enzyme activities in the plant material available, so that a determination and quantification of the enzyme activities within the linear measurement range are possible. Typically, the extraction buffer can consist of 50 mM HEPES-KOH (pH 7.4), 10 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.1% (v/v) Triton X-100, 2 mM ε-aminocaproic acid, 10% glycerol, 5 mM $KHCO_3$. 2 mM DTT and 0.5 mM PMSF are added shortly before the extraction.

The activity of the D-1-deoxyxylulose-5-phosphate reductoisomerase (DXR) is measured in a buffer consisting of 100 mM Tris-HCl (pH 7.5), 1 mM $MnCl_2$, 0.3 mM NADPH and 0.3 mM 1-deoxy-D-xylulose-4-phosphate, which can be synthesized, for example enzymatically (Kuzuyama, Takahashi, Watanabe and Seto: Tetrahedon letters 39 (1998) 4509-4512). The reaction is started by adding the plant extract. The reaction volume can typically be 0.2 to 0.5 ml; incubation is carried out at 37°C over 30-60 minutes. During this time, the oxidation of NADPH is monitored photometrically at 340 nm.

Isopentenyl-diphosphate Δ-isomerase activity is understood as meaning the enzyme activity of an isopentenyl-diphosphate Δ-isomerase.

An isopentenyl-diphosphate Δ-isomerase is understood as meaning a protein with the enzymatic activity of converting isopentenyl diphosphate into dimethylallyl phosphate.

Accordingly, isopentenyl-diphosphate Δ-isomerase activity is understood as meaning the amount of isopentenyl diphosphate converted, or the amount of dimethylallyl phosphate formed, by the protein isopentenyl-diphosphate Δ-isomerase within a certain period of time.

Thus, in the case of an increased isopentenyl-diphosphate Δ-isomerase activity in comparison with the wild type, the amount of isopentenyl diphosphate converted, or the amount of dimethylallyl phosphate formed, by the protein isopentenyl-diphosphate Δ-isomerase within a certain period of time is increased in comparison with the wild type.

By preference, this increase of isopentenyl-diphosphate Δ-isomerase activity amounts to at least 5%, further preferably to at least 20%, further preferably to at least 50%, further preferably to at least 100%, more preferably to at least 300%, even more preferably to at least 500%, in particular to at least

600%, of the isopentenyl-diphosphate Δ-isomerase activity of the wild type.

The isopentenyl-diphosphate Δ-isomerase activity in genetically modified plants according to the invention and in wild-type, or reference, plants is preferably determined under the following conditions:

Frozen plant material is homogenized by thoroughly crushing in liquid nitrogen and extracted with an extraction buffer in a ratio of from 1:1 to 1:20. The ratio in question depends on the enzyme activities in the plant material available, so that a determination and quantification of the enzyme activities within the linear measurement range are possible. Typically, the extraction buffer can consist of 50 mM HEPES-KOH (pH 7.4), 10 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.1% (v/v) Triton X-100, 2 mM ε-aminocaproic acid, 10% glycerol, 5 mM $KHCO_3$. 2 mM DTT and 0.5 mM PMSF are added shortly before the extraction.

Activity determinations of the isopentenyl-diphosphate isomerase (IPP isomerase) can be carried out by the method proposed by Fraser and coworkers (Fraser, Römer, Shipton, Mills, Kiano, Misawa, Drake, Schuch and Bramley: Evaluation of transgenic tomato plants expressing an additional phytoene synthase in a fruit-specific manner; Proc. Natl. Acad. Sci. USA 99 (2002), 1092-1097, based on Fraser, Pinto, Holloway und Bramley, Plant Journal 24 (2000), 551-558). For the enzyme measurements, incubations are carried out with 0.5 μCi (1-^{14}C)-IPP (isopentenyl pyrophosphate) (56 mCi/mmol, Amersham plc) as substrate in 0.4 M Tris-HCl (pH 8.0) with 1 mM DTT, 4 mM $MgCl_2$, 6 mM Mn Cl_2, 3 mM ATP, 0.1% Tween 60, 1 mM potassium fluoride in a volume of approximately 150-500 μl. Extracts are mixed with buffer (for example in the ratio 1:1) and incubated for at least 5 hours at 28°C. Thereafter, approximately 200 μl of methanol are added, and an acid hydrolysis is carried out for approximately 1 hour at 37°C by addition of concentrated hydrochloric acid (final concentration 25%). Thereafter, the mixture is extracted twice (in each case 500 μl) with petroleum ether (treated with 10% diethyl ether). The radioactivity in an aliquot of the hyperphase is determined by means of scintillation counter. The specific enzyme activity can be determined at a short incubation time of 5 minutes since short reaction times suppress the formation of by-products of the reaction (see Lützow and Beyer: The isopentenyl-diphosphate Δ-isomerase and its relation to the phytoene synthase complex in daffodil chromoplasts; Biochim. Biophys. Acta 959 (1988), 118-126)

Geranyl-diphosphate synthase activity is understood as meaning the enzyme activity of a geranyl-diphosphate synthase.

A geranyl-diphosphate synthase is understood as meaning a protein with the enzymatic activity of converting isopentenyl diphosphate and dimethylallyl phosphate into geranyl diphosphate.

Accordingly, geranyl-diphosphate synthase activity is understood as meaning the amount of isopentenyl diphosphate and/or dimethylallyl phosphate converted, or the amount of geranyl diphosphate formed, by the protein geranyl-diphosphate synthase within a certain period of time.

Thus, in the case of an increased geranyl-diphosphate synthase activity in comparison with the wild type, the amount of isopentenyl diphosphate and/or dimethylallyl phosphate converted, or the amount of geranyl diphosphate formed, by the protein geranyl-diphosphate synthase within a certain period of time is increased in comparison with the wild type.

By preference, this increase of geranyl-diphosphate synthase activity amounts to at least 5%, further preferably to at least 20%, further preferably to at least 50%, further preferably to at least 100%, more preferably to at least 300%, even more preferably to at least 500%, in particular to at least 600%, of the geranyl-diphosphate synthase activity of the wild type.

The geranyl-diphosphate synthase activity in genetically modified plants according to the invention and in wild-type, or reference, plants is preferably determined under the following conditions:

Frozen plant material is homogenized by thoroughly crushing in liquid nitrogen and extracted with an extraction buffer in a ratio of from 1:1 to 1:20. The ratio in question depends on the enzyme activities in the plant material available, so that a determination and quantification of the enzyme activities within the linear measurement range are possible. Typically, the extraction buffer can consist of 50 mM HEPES-KOH (pH 7.4), 10 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.1% (v/v) Triton X-100, 2 mM ε-aminocaproic acid, 10% glycerol, 5 mM $KHCO_3$. 2 mM DTT and 0.5 mM PMSF are added shortly before the extraction.

The activity of the geranyl-diphosphate synthase (GPP synthase) can be determined in 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM $MnCl_2$, 2 mM DTT, 1 mM ATP, 0.2% Tween-20, 5 µM (^{14}C)-IPP and 50 µM DMAPP (dimethylallyl pyrophosphate) after the addition of plant extract (by the method of Bouvier, Suire, d'Harlingue, Backhaus

and Camara: Molecular cloning of geranyl diphosphate synthase and compartmentation of monoterpene synthesis in plant cells, Plant Journal 24 (2000), 241-252). After incubation of, for example, 2 hours at 37°C, the reaction products are dephosphorylated (by the method of Koyama, Fuji and Ogura: Enzymatic hydrolysis of polyprenyl pyrophosphates, Methods Enzymol. 110 (1985), 153-155) and analyzed by means of thin-layer chromatography and measuring the incorporated radioactivity (Dogbo, Bardat, Quennemet and Camara: Metabolism of plastid terpenoids: In vitro inhibition of phytoene synthesis by phenethyl pyrophosphate derivates, FEBS Letters 219 (1987) 211-215).

Farnesyl-diphosphate synthase activity is understood as meaning the enzyme activity of a farnesyl-diphosphate synthase.

A farnesyl-diphosphate synthase is understood as meaning a protein with the enzymatic activity of converting dimethylallyl diphosphate and isopentenyl diphosphate into farnesyl diphosphate.

Accordingly, farnesyl-diphosphate synthase activity is understood as meaning the amount of dimethylallyl diphosphate and/or isopentenyl diphosphate converted, or the amount of farnesyl diphosphate formed, by the protein farnesyl-diphosphate synthase within a certain period of time.

Thus, in the case of an increased farnesyl-diphosphate synthase activity in comparison with the wild type, the amount of dimethylallyl diphosphate and/or isopentenyl diphosphate converted, or the amount of farnesyl diphosphate formed, by the protein farnesyl-diphosphate synthase within a certain period of time is increased in comparison with the wild type.

By preference, this increase of farnesyl-diphosphate synthase activity amounts to at least 5%, further preferably to at least 20%, further preferably to at least 50%, further preferably to at least 100%, more preferably to at least 300%, even more preferably to at least 500%, in particular to at least 600%, of the farnesyl-diphosphate synthase activity of the wild type.

The farnesyl-diphosphate synthase activity in genetically modified plants according to the invention and in wild-type, or reference, plants is preferably determined under the following conditions:

Frozen plant material is homogenized by thoroughly crushing in liquid nitrogen and extracted with an extraction buffer in a ratio of from 1:1 to 1:20. The ratio in question depends on the enzyme activities in the plant material available, so that a determination and quantification of the enzyme activities within the linear measurement range are possible. Typically, the extraction buffer can consist of 50 mM HEPES-KOH (pH 7.4), 10 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.1% (v/v) Triton X-100, 2 mM ε-aminocaproic acid, 10% glycerol, 5 mM $KHCO_3$. 2 mM DTT and 0.5 mM PMSF are added shortly before the extraction.

The farnesyl-pyrophosphate synthase (FPP synthase) activity can be determined by a protocol of Joly and Edwards (Journal of Biological Chemistry 268 (1993), 26983-26989). According to this protocol, the enzyme activity is measured in a buffer consisting of 10 mM HEPES (pH 7.2), 1 mM $MgCl_2$, 1 mM dithiothreitol, 20 µM geranyl pyrophosphate and 40 µM (1-^{14}C)-isopentenyl pyrophosphate (4 Ci/mmol). The reaction mixture is incubated at 37°C; the reaction is quenched by addition of 2.5 N HCl (in 70% ethanol supplemented with 19 µg/ml farnesol). The reaction products are thus hydrolyzed within 30 minutes by acid hydrolysis at 37°C. The mixture is neutralized by addition of 10% NaOH and extracted by shaking with hexane. An aliquot of the hexane phase can be measured for determining the incorporated radioactivity by means of scintillation counter.

As an alternative, the reaction products obtained after the incubation of plant extract and radiolabeled IPP can be separated by means of thin-layer chromatography (Silica-Gel SE60, Merck) in benzene/methanol (9:1). Radiolabeled products are eluted and the radioactivity is determined (by the method of Gaffe, Bru, Causse, Vidal, Stamitti-Bert, Carde and Gallusci: LEFPS1, a tomato farnesyl pyrophosphate gene highly expressed during early fruit development; Plant Physiology 123 (2000) 1351-1362).

Geranylgeranyl-diphosphate synthase activity is understood as meaning the enzyme activity of a geranylgeranyl-diphosphate synthase.

A geranylgeranyl-diphosphate synthase is understood as meaning a protein with the enzymatic activity of converting farnesyl diphosphate and isopentenyl diphosphate into geranylgeranyl diphosphate.

Accordingly, geranylgeranyl-diphosphate synthase activity is understood as meaning the amount of farnesyl diphosphate and/or isopentenyl diphosphate converted, or the amount of geranyl-

geranyl diphosphate formed, by the protein geranylgeranyl-diphosphate synthase within a certain period of time.

Thus, in the case of an increased geranylgeranyl-diphosphate synthase activity in comparison with the wild type, the amount of farnesyl diphosphate and/or isopentenyl diphosphate converted, or the amount of geranylgeranyl diphosphate formed, by the protein geranylgeranyl-diphosphate synthase within a certain period of time is increased in comparison with the wild type.

By preference, this increase of geranylgeranyl-diphosphate synthase activity amounts to at least 5%, further preferably to at least 20%, further preferably to at least 50%, further preferably to at least 100%, more preferably to at least 300%, even more preferably to at least 500%, in particular to at least 600%, of the geranylgeranyl-diphosphate synthase activity of the wild type.

The geranylgeranyl-diphosphate synthase activity in genetically modified plants according to the invention and in wild-type, or reference, plants is preferably determined under the following conditions:

Frozen plant material is homogenized by thoroughly crushing in liquid nitrogen and extracted with an extraction buffer in a ratio of from 1:1 to 1:20. The ratio in question depends on the enzyme activities in the plant material available, so that a determination and quantification of the enzyme activities within the linear measurement range are possible. Typically, the extraction buffer can consist of 50 mM HEPES-KOH (pH 7.4), 10 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.1% (v/v) Triton X-100, 2 mM ε-aminocaproic acid, 10% glycerol, 5 mM $KHCO_3$. 2 mM DTT and 0.5 mM PMSF are added shortly before the extraction.

Measurements of the geranylgeranyl-pyrophosphate synthase (GGPP synthase) activity can be carried out by the method described by Dogbo and Camara (in Biochim. Biophys. Acta 920 (1987), 140-148: Purification of isopentenyl pyrophosphate isomerase and geranylgeranyl pyrophosphate synthase from Capsicum chromoplasts by affinity chromatography). To this end, plant extract is added to a buffer (50 mM Tris-HCl (pH 7.6), 2 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM dithiothreitol, (1-^{14}C)-IPP (0.1 µCi, 10 µM), 15 µM DMAPP, GPP or FPP) with a total volume of approximately 200 µl. The incubation can be carried out for 1-2 hours (or longer) at 30°C. The reaction is quenched by addition of 0.5 ml of ethanol and 0.1 ml of 6N HCl. After incubation for 10 minutes at 37°C, the reaction mixture is neutralized with 6N NaOH, mixed with 1 ml of water and

extracted by shaking with 4 ml of diethyl ether. The amount of radioactivity is determined in an aliquot (for example 0.2 ml) of the ether phase by means of scintillation counting. As an alternative, the radiolabeled prenyl alcohols can be subjected to acid hydrolysis and then extracted by shaking in ether and separated by means of HPLC (25 cm column Spherisorb ODS-1, 5 µm; elution with methanol/water (90:10; v/v) at a flow rate of 1 ml/min) and determined quantitatively by means of radioactivity monitoring (by the method of Wiedemann, Misawa and Sandmann: Purification and enzymatic characterization of the geranylgeranyl pyrophosphate synthase from Erwinia uredovora after expression in Escherichia coli).

Phytoene synthase activity is understood as meaning the enzyme activity of a phytoene synthase.

In particular, a phytoene synthase is understood as meaning a protein with the enzymatic activity of converting geranylgeranyl diphosphate into phytoene.

Accordingly, phytoene synthase activity is understood as meaning the amount of geranylgeranyl diphosphate converted, or the amount of phytoene formed, by the protein phytoene synthase within a certain period of time.

Thus, in the case of an increased phytoene synthase activity in comparison with the wild type, the amount of geranylgeranyl diphosphate converted, or the amount of phytoene formed, by the protein phytoene synthase within a certain period of time is increased in comparison with the wild type.

By preference, this increase of phytoene synthase activity amounts to at least 5%, further preferably to at least 20%, further preferably to at least 50%, further preferably to at least 100%, more preferably to at least 300%, even more preferably to at least 500%, in particular to at least 600%, of the phytoene synthase activity of the wild type.

The phytoene synthase activity in genetically modified plants according to the invention and in wild-type, or reference, plants is preferably determined under the following conditions:

Frozen plant material is homogenized by thoroughly crushing in liquid nitrogen and extracted with an extraction buffer in a ratio of from 1:1 to 1:20. The ratio in question depends on the enzyme activities in the plant material available, so that a determination and quantification of the enzyme activities within

the linear measurement range are possible. Typically, the extraction buffer can consist of 50 mM HEPES-KOH (pH 7.4), 10 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.1% (v/v) Triton X-100, 2 mM ε-aminocaproic acid, 10% glycerol, 5 mM $KHCO_3$. 2 mM DTT and 0.5 mM PMSF are added shortly before the extraction.

Phytoene synthase (PSY) activity determinations can be carried out by the method proposed by Fraser and coworkers (Fraser, Romer, Shipton, Mills, Kiano, Misawa, Drake, Schuch and Bramley: Evaluation of transgenic tomato plants expressing an additional phytoene synthase in a fruit-specific manner; Proc. Natl. Acad. Sci. USA 99 (2002), 1092-1097, based on Fraser, Pinto, Holloway and Bramley, Plant Journal 24 (2000) 551-558). For enzyme measurements, incubations are carried out with (^{3}H)geranylgeranyl pyrophosphate (15 mCi/mM, American Radiolabeled Chemicals, St. Louis) as substrate in 0.4 M Tris-HCl (pH 8.0) with 1 mM DTT, 4 mM $MgCl_2$, 6 mM Mn Cl_2, 3 mM ATP, 0.1% Tween 60, 1 mM potassium fluoride. Plant extracts are mixed with buffer, for example 295 µl of buffer with extract in a total volume of 500 µl. The mixture is incubated for at least 5 hours at 28°C. Thereafter, phytoene is extracted by shaking twice (in each case 500 µl) with chloroform. The radiolabeled phytoene, which has formed during the reaction, is separated by means of thin-layer chromatography on silica plates in methanol/water (95:5; v/v). Phytoene can be identified in an iodine-enriched atmosphere (by heating a few iodine crystals) on the silica plates. A phytoene standard is used as reference. The amount of radiolabeled product is determined by measuring in the scintillation counter. As an alternative, phytoene can also be determined quantitatively by means of HPLC equipped with a radioactivity detector (Fraser, Albrecht and Sandmann: Development of high performance liquid chromatographic systems for the separation of radiolabeled carotenes and precursors formed in specific enzymatic reactions; J. Chromatogr. 645 (1993) 265-272).

Phytoene desaturase activity is understood as meaning the enzyme activity of a phytoene desaturase.

A phytoene desaturase is understood as meaning a protein with the enzymatic activity of converting phytoene into phytofluene and/or phytofluene into ζ-carotene (zetacarotene).

Accordingly, phytoene desaturase activity is understood as meaning the amount of phytoene or phytofluene converted, or the amount of phytofluene or ζ-carotene formed, by the protein phytoene desaturase within a certain period of time.

Thus, in the case of an increased phytoene desaturase activity in comparison with the wild type, the amount of phytoene or phytofluene converted, or the amount of phytofluene or ζ-carotene formed, by the protein phytoene desaturase within a certain period of time is increased in comparison with the wild type.

By preference, this increase of phytoene desaturase activity amounts to at least 5%, further preferably to at least 20%, further preferably to at least 50%, further preferably to at least 100%, more preferably to at least 300%, even more preferably to at least 500%, in particular to at least 600%, of the phytoene desaturase activity of the wild type.

The phytoene desaturase activity in genetically modified plants according to the invention and in wild-type, or reference, plants is preferably determined under the following conditions:

Frozen plant material is homogenized by thoroughly crushing in liquid nitrogen and extracted with an extraction buffer in a ratio of from 1:1 to 1:20. The ratio in question depends on the enzyme activities in the plant material available, so that a determination and quantification of the enzyme activities within the linear measurement range are possible. Typically, the extraction buffer can consist of 50 mM HEPES-KOH (pH 7.4), 10 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.1% (v/v) Triton X-100, 2 mM ε-aminocaproic acid, 10% glycerol, 5 mM $KHCO_3$. 2 mM DTT and 0.5 mM PMSF are added shortly before the extraction.

The phytoene desaturase (PDS) activity can be measured on the basis of incorporation of radiolabeled (^{14}C)-phytoene in unsaturated carotene (by the method of Römer, Fraser, Kiano, Shipton, Misawa, Schuch and Bramley: Elevation of the provitamin A content of transgenic tomato plants; Nature Biotechnology 18 (2000) 666-669). Radiolabeled phytoene can be synthetized by the method of Fraser (Fraser, De la Rivas, Mackenzie, Bramley: Phycomyces blakesleanus CarB mutants: their use in assays of phytoene desaturase; Phytochemistry 30 (1991), 3971-3976). Membranes of plastids of the target tissue can be incubated with 100 mM MES buffer (pH 6.0) supplemented with 10 mM $MgCl_2$ and 1 mM dithiothreitol in a total volume of 1 ml. (^{14}C)-Phytoene (approximately 100 000 decays/minute per incubation) dissolved in acetone is added; the acetone concentration should not exceed 5% (v/v). This mixture is incubated with shaking in the dark at 28°C for approximately 6 to 7 hours. Thereafter, pigments are extracted three times with approximately 5 ml petroleum ether

(treated with 10% diethyl ether) and separated and determined quantitatively by means of HPLC.

As an alternative, the phytoene desaturase activity can be measured by the method of Fraser et al. (Fraser, Misawa, Linden, Yamano, Kobayashi and Sandmann: Expression in Escherichia coli, purification, and reactivation of the recombinant Erwinia uredovora phytoene desaturase, Journal of Biological Chemistry 267 (1992), 9891-9895).

Zeta-carotene desaturase activity is understood as meaning the enzyme activity of a zeta-carotene desaturase.

A zeta-carotene desaturase is understood as meaning a protein with the enzymatic activity of converting ζ-carotene into neurosporin and/or neurosporin into lycopene.

Accordingly, zeta-carotene desaturase activity is understood as meaning the amount of ζ-carotene or neurosporin converted, or the amount of neurosporin or lycopene formed, by the protein zeta-carotene desaturase within a certain period of time.

Thus, in the case of an increased zeta-carotene desaturase activity in comparison with the wild type, the amount of ζ-carotene or neurosporin converted, or the amount of neurosporin or lycopene formed, by the protein zeta-carotene desaturase within a certain period of time is increased in comparison with the wild type.

By preference, this increase of zeta-carotene desaturase activity amounts to at least 5%, further preferably to at least 20%, further preferably to at least 50%, further preferably to at least 100%, more preferably to at least 300%, even more preferably to at least 500%, in particular to at least 600%, of the zeta-carotene desaturase activity of the wild type.

The zeta-carotene desaturase activity in genetically modified plants according to the invention and in wild-type, or reference, plants is preferably determined under the following conditions:

Frozen plant material is homogenized by thoroughly crushing in liquid nitrogen and extracted with an extraction buffer in a ratio of from 1:1 to 1:20. The ratio in question depends on the enzyme activities in the plant material available, so that a determination and quantification of the enzyme activities within the linear measurement range are possible. Typically, the extraction buffer can consist of 50 mM HEPES-KOH (pH 7.4), 10 mM

$MgCl_2$, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.1% (v/v) Triton X-100, 2 mM ε-aminocaproic acid, 10% glycerol, 5 mM $KHCO_3$. 2 mM DTT and 0.5 mM PMSF are added shortly before the extraction.

Analyses for determining the ξ-carotene desaturase (ZDS-desaturase) activity can be carried out in 0.2 M potassium phosphate (pH 7.8, buffer volume approximately 1 ml). The relevant analytical method has been published by Breitenbach and coworkers (Breitenbach, Kuntz, Takaichi and Sandmann: Catalytic properties of an expressed and purified higher plant type ξ-carotene desaturase from Capsicum annuum; European Journal of Biochemistry. 265(1):376-383, 1999 Oct). Each batch of the analysis comprises 3 mg phosphytidylcholine which is suspended in 0.4 M potassium phosphate buffer (pH 7.8), 5 µg of ξ-carotene or neurosporin, 0.02% of butylhydroxytoluene, 10 µl of decyl-plastoquinone (1 mM methanolic stock solution) and plant extract. The volume of the plant extract must be adapted to the amount of ZDS-desaturase activity present in order to make possible quantitative determinations in a linear measurement range. Incubations are typically carried out for approximately 17 hours with vigorous shaking (200 revolutions/minute) at approximately 28°C in the dark. Carotenoids are extracted by addition of 4 ml of acetone at 50°C for 10 minutes with shaking. The carotenoids are transferred from this mixture into a petroleum ether phase (supplemented with 10% diethyl ether). The diethyl ether/petroleum ether phase is evaporated under nitrogen, the carotenoids are redissolved in 20 µl, and separated and quantitatively determined by means of HPLC.

crtISO activity is understood as meaning the enzyme activity of a crtISO protein.

A crtISO protein is understood as meaning a protein with the enzymatic activity of converting 7,9,7',9'-tetra-cis-lycopene into all-trans-lycopene.

Accordingly, crtISO activity is understood as meaning the amount of 7,9,7',9'-tetra-cis-lycopene converted, or the amount of all-trans-lycopene formed, by the protein crtISO within a certain period of time.

Thus, in the case of an increased crtISO activity in comparison with the wild type, the amount of 7,9,7',9'-tetra-cis-lycopene converted, or the amount of all-trans-lycopene formed, by the crtISO protein within a certain period of time is increased in comparison with the wild type.

By preference, this increase of the crtISO activity amounts to at least 5%, further preferably to at least 20%, further preferably to at least 50%, further preferably to at least 100%, more preferably to at least 300%, even more preferably to at least 500%, in particular to at least 600%, of the crtISO activity of the wild type.

FtsZ activity is understood as meaning physiological activity of an FtsZ protein.

An FtsZ protein is understood as meaning a protein with an activity which promotes cell division and plastid division and which has homologies with tubulin proteins.

MinD activity is understood as meaning the physiological activity of a MinD protein.

A MinD protein is understood as meaning a protein which plays a multifunctional role in cell division. It is a membrane-associated ATPase and can show an oscillating movement from pole to pole within the cell.

Moreover, increasing the activity of enzymes of the non-mevalonate pathway can lead to a further increase of the desired ketocarotenoid end product. Examples are 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase, 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase and 2-C-methyl-D-erythritol-2,4-cyclodiphoshate synthase. By modifying gene expression of the genes in question, the activity of the abovementioned enzymes can be increased. The altered concentrations of the relevant proteins can be detected by standard techniques using antibodies and suitable blotting techniques.

Increasing the HMG-CoA reductase activity and/or (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity and/or 1-deoxy-D-xylose-5-phosphate synthase activity and/or 1-deoxy-D-xylose-5-phosphate reductoisomerase activity and/or isopentenyl-diphosphate Δ-isomerase activity and/or geranyl-diphosphate synthase activity and/or farnesyl-diphosphate synthase activity and/or geranylgeranyl-diphosphate synthase activity and/or phytoene synthase activity and/or phytoene desaturase activity and/or zeta-carotene desaturase activity and/or crtISO activity and/or FtsZ activity and/or MinD activity can be effected in different ways, for example by eliminating inhibiting regulatory mechanisms at the expression and protein level, or by increasing the gene expression of nucleic acids encoding an HMG-CoA reductase and/or nucleic acids encoding an

(E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase and/or nucleic acids encoding a 1-deoxy-D-xylose-5-phosphate synthase and/or nucleic acids encoding a 1-deoxy-D-xylose-5-phosphate reductoisomerase and/or nucleic acids encoding an isopentenyl-diphosphate Δ-isomerase and/or nucleic acids encoding a geranyl-diphosphate synthase and/or nucleic acids encoding a farnesyl-diphosphate synthase and/or nucleic acids encoding a geranylgeranyl-diphosphate synthase and/or nucleic acids encoding a phytoene synthase and/or nucleic acids encoding a phytoene desaturase and/or nucleic acids encoding a zeta-carotene desaturase and/or nucleic acids encoding a crtISO protein and/or nucleic acids encoding an FtsZ protein and/or nucleic acids encoding a MinD protein in comparison with the wild type.

Increasing the gene expression of nucleic acids encoding an HMG-CoA reductase and/or nucleic acids encoding an (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase and/or nucleic acids encoding a 1-deoxy-D-xylose-5-phosphate synthase and/or nucleic acids encoding a 1-deoxy-D-xylose-5-phosphate reductoisomerase and/or nucleic acids encoding an isopentenyl-diphosphate Δ-isomerase and/or nucleic acids encoding a geranyl-diphosphate synthase and/or nucleic acids encoding a farnesyl-diphosphate synthase and/or nucleic acids encoding a geranylgeranyl-diphosphate synthase and/or nucleic acids encoding a phytoene synthase and/or nucleic acids encoding a phytoene desaturase and/or nucleic acids encoding a zeta-carotene desaturase and/or nucleic acids encoding a crtISO protein and/or nucleic acids encoding an FtsZ protein and/or nucleic acids encoding a MinD protein in comparison with the wild type can likewise be effected in different ways, for example by inducing the HMG-CoA reductase gene and/or (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase gene and/or 1-deoxy-D-xylose-5-phosphate synthase gene and/or 1-deoxy-D-xylose-5-phosphate reductoisomerase gene and/or isopentenyl-diphosphate Δ-isomerase gene and/or geranyl-diphosphate synthase gene and/or farnesyl-diphosphate synthase gene and/or geranylgeranyl-diphosphate synthase gene and/or phytoene synthase gene and/or phytoene desaturase gene and/or zeta-carotene desaturase gene and/or crtISO gene and/or FtsZ gene and/or MinD gene by activators or by introducing one or more copies of the HMG-CoA reductase gene and/or (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase gene and/or 1-deoxy-D-xylose-5-phosphate synthase gene and/or 1-deoxy-D-xylose-5-phosphate reductoisomerase gene and/or isopentenyl-diphosphate Δ-isomerase gene and/or geranyl-diphosphate synthase gene and/or farnesyl-diphosphate synthase gene and/or geranylgeranyl-diphosphate synthase gene

and/or phytoene synthase gene and/or phytoene desaturase gene and/or zeta-carotene desaturase gene and/or crtISO gene and/or FtsZ gene and/or MinD gene, i.e. by introducing at least one nucleic acid encoding an HMG-CoA reductase and/or at least one nucleic acid encoding an (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase and/or at least one nucleic acid encoding a 1-deoxy-D-xylose-5-phosphate synthase and/or at least one nucleic acid encoding a 1-deoxy-D-xylose-5-phosphate reductoisomerase and/or at least one nucleic acid encoding an isopentenyl-diphosphate Δ-isomerase and/or at least one nucleic acid encoding a geranyl-diphosphate synthase and/or at least one nucleic acid encoding a farnesyl-diphosphate synthase and/or at least one nucleic acid encoding a geranylgeranyl-diphosphate synthase and/or at least one nucleic acid encoding a phytoene synthase and/or at least one nucleic acid encoding a phytoene desaturase and/or at least one nucleic acid encoding a zeta-carotene desaturase and/or at least one nucleic acid encoding a crtISO protein and/or at least one nucleic acid encoding an FtsZ protein and/or at least one nucleic acid encoding a MinD protein into the plant.

In accordance with the invention, increasing the gene expression of a nucleic acid encoding an HMG-CoA reductase and/or (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase and/or 1-deoxy-D-xylose-5-phosphate synthase and/or 1-deoxy-D-xylose-5-phosphate reductoisomerase and/or isopentenyl-diphosphate Δ-isomerase and/or geranyl-diphosphate synthase and/or farnesyl-diphosphate synthase and/or geranylgeranyl-diphosphate synthase and/or phytoene synthase and/or phytoene desaturase and/or zeta-carotene desaturase and/or a crtISO protein and/or FtsZ protein and/or MinD protein is also understood as meaning the manipulation of the expression of the plant's homologous, endogenous HMG-CoA reductase and/or (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase and/or 1-deoxy-D-xylose-5-phosphate synthase and/or 1-deoxy-D-xylose-5-phosphate reductoisomerase and/or isopentenyl-diphosphate Δ-isomerase and/or geranyl-diphosphate synthase and/or farnesyl-diphosphate synthase and/or geranylgeranyl-diphosphate synthase and/or phytoene synthase and/or phytoene desaturase and/or zeta-carotene desaturase and/or of the plant's homologous crtISO protein and/or FtsZ protein and/or MinD protein.

This can be achieved for example by modifying the corresponding promoter DNA sequence. Such a modification, which results in an increased expression rate of the gene, can be effected for example by deleting or inserting DNA sequences.

In a preferred embodiment, increasing the gene expression of a nucleic acid encoding an HMG-CoA reductase and/or increasing the gene expression of a nucleic acid encoding an (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase and/or increasing the gene expression of a nucleic acid encoding a 1-deoxy-D-xylose-5-phosphate synthase and/or increasing the gene expression of a nucleic acid encoding a 1-deoxy-D-xylose-5-phosphate reductoisomerase and/or increasing the gene expression of a nucleic acid encoding an isopentenyl-diphosphate Δ-isomerase and/or increasing the gene expression of a nucleic acid encoding a geranyl-diphosphate synthase and/or increasing the gene expression of a nucleic acid encoding a farnesyl-diphosphate synthase and/or increasing the gene expression of a nucleic acid encoding a geranylgeranyl-diphosphate synthase and/or increasing the gene expression of a nucleic acid encoding a phytoene synthase and/or increasing the gene expression of a nucleic acid encoding a phytoene desaturase and/or increasing the gene expression of a nucleic acid encoding a zeta-carotene desaturase and/or increasing the gene expression of a nucleic acid encoding a crtISO protein and/or increasing the gene expression of a nucleic acid encoding a FtsZ protein and/or increasing the gene expression of a nucleic acid encoding a MinD protein is effected by introducing at least one nucleic acid encoding an HMG-CoA reductase and/or by introducing at least one nucleic acid encoding an (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase and/or by introducing at least one nucleic acid encoding a 1-deoxy-D-xylose-5-phosphate synthase and/or by introducing at least one nucleic acid encoding a 1-deoxy-D-xylose-5-phosphate reductoisomerase and/or by introducing at least one nucleic acid encoding an isopentenyl-diphosphate Δ-isomerase and/or by introducing at least one nucleic acid encoding a geranyl-diphosphate synthase and/or by introducing at least one nucleic acid encoding a farnesyl-diphosphate synthase and/or by introducing at least one nucleic acid encoding a geranylgeranyl-diphosphate synthase and/or by introducing at least one nucleic acid encoding a phytoene synthase and/or by introducing at least one nucleic acid encoding a phytoene desaturase and/or by introducing at least one nucleic acid encoding a zeta-carotene desaturase and/or by introducing at least one nucleic acid encoding a crtISO protein and/or by introducing at least one

nucleic acid encoding an FtsZ protein and/or by introducing at least one nucleic acid encoding a MinD protein into the plant.

To this end, any HMG-CoA reductase gene and/or (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase gene and/or 1-deoxy-D-xylose-5-phosphate synthase gene and/or 1-deoxy-D-xylose-5-phosphate reductoisomerase gene and/or isopentenyl-diphosphate Δ-isomerase gene and/or geranyl-diphosphate synthase gene and/or farnesyl-diphosphate synthase gene and/or geranylgeranyl-diphosphate synthase gene and/or phytoene synthase gene and/or phytoene desaturase gene and/or zeta-carotene desaturase gene and/or crtISO gene and/or FtsZ gene and/or MinD gene can be used in principle.

In the case of genomic HMG-CoA reductase sequences and/or (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase sequences and/or 1-deoxy-D-xylose-5-phosphate synthase sequences and/or 1-deoxy-D-xylose-5-phosphate reductoisomerase sequences and/or isopentenyl-diphosphate Δ-isomerase sequences and/or geranyl-diphosphate synthase sequences and/or farnesyl-diphosphate synthase sequences and/or geranylgeranyl-diphosphate synthase sequences and/or phytoene synthase sequences and/or phytoene desaturase sequences and/or zeta-carotene desaturase sequences and/or crtISO sequences and/or FtsZ sequences and/or MinD sequences from eukaryotic sources, which comprise introns, it is preferred to use ready-processed nucleic acid sequences, such as the corresponding cDNAs, in the event that the host plant is not capable, or cannot be made capable, of expressing the proteins in question.

Thus, in this preferred embodiment, at least one further HMG-CoA reductase gene and/or (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase gene and/or 1-deoxy-D-xylose-5-phosphate synthase gene and/or 1-deoxy-D-xylose-5-phosphate reductoisomerase gene and/or isopentenyl-diphosphate Δ-isomerase gene and/or geranyl-diphosphate synthase gene and/or farnesyl-diphosphate synthase gene and/or geranylgeranyl-diphosphate synthase gene and/or phytoene synthase gene and/or phytoene desaturase gene and/or zeta-carotene desaturase gene and/or crtISO gene and/or FtsZ gene and/or MinD gene is present in the preferred transgenic plants according to the invention in comparison with the wild type.

In this preferred embodiment, the genetically modified plant shows, for example, at least one exogenous nucleic acid encoding an HMG-CoA reductase or at least two endogenous nucleic acids encoding an HMG-CoA reductase and/or at least one exogenous

nucleic acid encoding an (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase or at least two endogenous nucleic acids encoding an (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase and/or at least one exogenous nucleic acid encoding a 1-deoxy-D-xylose-5-phosphate synthase or at least two endogenous nucleic acids encoding a 1-deoxy-D-xylose-5-phosphate synthase and/or at least one exogenous nucleic acid encoding a 1-deoxy-D-xylose-5-phosphate reductoisomerase or at least two endogenous nucleic acids encoding a 1-deoxy-D-xylose-5-phosphate reductoisomerase and/or at least one exogenous nucleic acid encoding an isopentenyl-diphosphate Δ-isomerase or at least two endogenous nucleic acids encoding an isopentenyl-diphosphate Δ-isomerase and/or at least one exogenous nucleic acid encoding a geranyl-diphosphate synthase or at least two endogenous nucleic acids encoding a geranyl-diphosphate synthase and/or at least one exogenous nucleic acid encoding a farnesyl-diphosphate synthase or at least two endogenous nucleic acids encoding a farnesyl-diphosphate synthase and/or at least one exogenous nucleic acid encoding a geranylgeranyl-diphosphate synthase or at least two endogenous nucleic acids encoding a geranylgeranyl-diphosphate synthase and/or at least one exogenous nucleic acid encoding a phytoene synthase or at least two endogenous nucleic acids encoding a phytoene synthase and/or at least one exogenous nucleic acid encoding a phytoene desaturase or at least two endogenous nucleic acids encoding a phytoene desaturase and/or at least one exogenous nucleic acid encoding a zeta-carotene desaturase or at least two endogenous nucleic acids encoding a zeta-carotene desaturase and/or at least one exogenous nucleic acid encoding a crtISO protein or at least two endogenous nucleic acids encoding a crtISO protein and/or at least one exogenous nucleic acid encoding an FtsZ protein or at least two endogenous nucleic acids encoding an FtsZ protein and/or at least one exogenous nucleic acid encoding a MinD protein or at least two endogenous nucleic acids encoding a MinD protein.

Examples of HMG-CoA reductase genes are:

a nucleic acid encoding an HMG-CoA reductase from Arabidopsos thaliana, Accession NM_106299; (nucleic acid: SEQ ID NO: 99, protein: SEQ ID NO: 100),

and further HMG-CoA reductase genes from other organisms with the following accession numbers:

P54961, P54870, P54868, P54869, O02734, P22791, P54873, P54871, P23228, P13704, P54872, Q01581, P17425, P54874, P54839, P14891, P34135, O64966, P29057, P48019, P48020, P12683, P43256, Q9XEL8,

P34136, O64967, P29058, P48022, Q41437, P12684, Q00583, Q9XHL5, Q41438, Q9YAS4, O76819, O28538, Q9Y7D2, P54960, O51628, P48021, Q03163, P00347, P14773, Q12577, Q59468, P04035, O24594, P09610, Q58116, O26662, Q01237, Q01559, Q12649, O74164, O59469, P51639, Q10283, O08424, P20715, P13703, P13702, Q96UG4, Q8SQZ9, O15888, Q9TUM4, P93514, Q39628, P93081, P93080, Q944T9, Q40148, Q84MM0, Q84LS3, Q9Z9N4, Q9KLM0

Examples of (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase genes are:

a nucleic acid encoding an (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase from Arabidopsis thaliana (lytB/ISPH), ACCESSION AY168881, (nucleic acid: SEQ ID NO: 101, protein: SEQ ID NO:102),

and further (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase genes from other organisms with the following accession numbers:

T04781, AF270978_1, NP_485028.1, NP_442089.1, NP_681832.1, ZP_00110421.1, ZP_00071594.1, ZP_00114706.1, ISPH_SYNY3, ZP_00114087.1, ZP_00104269.1, AF398145_1, AF398146_1, AAD55762.1, AF514843_1, NP_622970.1, NP_348471.1, NP_562001.1, NP_223698.1, NP_781941.1, ZP_00080042.1, NP_859669.1, NP_214191.1, ZP_00086191.1, ISPH_VIBCH, NP_230334.1, NP_742768.1, NP_302306.1, ISPH_MYCLE, NP_602581.1, ZP_00026966.1, NP_520563.1, NP_253247.1, NP_282047.1, ZP_00038210.1, ZP_00064913.1, CAA61555.1, ZP_00125365.1, ISPH_ACICA, EAA24703.1, ZP_00013067.1, ZP_00029164.1, NP_790656.1, NP_217899.1, NP_641592.1, NP_636532.1, NP_719076.1, NP_660497.1, NP_422155.1, NP_715446.1, ZP_00090692.1, NP_759496.1, ISPH_BURPS, ZP_00129657.1, NP_215626.1, NP_335584.1, ZP_00135016.1, NP_789585.1, NP_787770.1, NP_769647.1, ZP_00043336.1, NP_242248.1, ZP_00008555.1, NP_246603.1, ZP_00030951.1, NP_670994.1, NP_404120.1, NP_540376.1, NP_733653.1, NP_697503.1, NP_840730.1, NP_274828.1, NP_796916.1, ZP_00123390.1, NP_824386.1, NP_737689.1, ZP_00021222.1, NP_757521.1, NP_390395.1, ZP_00133322.1, CAD76178.1, NP_600249.1, NP_454660.1, NP_712601.1, NP_385018.1, NP_751989.1

Examples of 1-deoxy-D-xylose-5-phosphate synthase genes are:

a nucleic acid encoding a 1-deoxy-D-xylose-5-phosphate synthase from Lycopersicon esculentum, ACCESSION #AF143812 (nucleic acid: SEQ ID NO:103, protein: SEQ ID NO: 104),

and further 1-deoxy-D-xylose-5-phosphate synthase genes from other organisms with the following accession numbers: AF143812_1, DXS_CAPAN, CAD22530.1, AF182286_1, NP_193291.1, T52289, AAC49368.1, AAP14353.1, D71420, DXS_ORYSA, AF443590_1, BAB02345.1, CAA09804.2, NP_850620.1, CAD22155.2, AAM65798.1, NP_566686.1, CAD22531.1, AAC33513.1, CAC08458.1, AAG10432.1, T08140, AAP14354.1, AF428463_1, ZP_00010537.1, NP_769291.1, AAK59424.1, NP_107784.1, NP_697464.1, NP_540415.1, NP_196699.1, NP_384986.1, ZP_00096461.1, ZP_00013656.1, NP_353769.1, BAA83576.1, ZP_00005919.1, ZP_00006273.1, NP_420871.1, AAM48660.1, DXS_RHOCA, ZP_00045608.1, ZP_00031686.1, NP_841218.1, ZP_00022174.1, ZP_00086851.1, NP_742690.1, NP_520342.1, ZP_00082120.1, NP_790545.1, ZP_00125266.1, CAC17468.1, NP_252733.1, ZP_00092466.1, NP_439591.1, NP_414954.1, NP_752465.1, NP_622918.1, NP_286162.1, NP_836085.1, NP_706308.1, ZP_00081148.1, NP_797065.1, NP_213598.1, NP_245469.1, ZP_00075029.1, NP_455016.1, NP_230536.1, NP_459417.1, NP_274863.1, NP_283402.1, NP_759318.1, NP_406652.1, DXS_SYNLE, DXS_SYNP7, NP_440409.1, ZP_00067331.1, ZP_00122853.1, NP_717142.1, ZP_00104889.1, NP_243645.1, NP_681412.1, DXS_SYNEL, NP_637787.1, DXS_CHLTE, ZP_00129863.1, NP_661241.1, DXS_XANCP, NP_470738.1, NP_484643.1, ZP_00108360.1, NP_833890.1, NP_846629.1, NP_658213.1, NP_642879.1, ZP_00039479.1, ZP_00060584.1, ZP_00041364.1, ZP_00117779.1, NP_299528.1

Examples of 1-deoxy-D-xylose-5-phosphate reductoisomerase genes are:

a nucleic acid encoding a 1-deoxy-D-xylose-5-phosphate reductoisomerase from Arabidopsis thaliana, ACCESSION #AF148852, (nucleic acid: SEQ ID NO: 105, protein: SEQ ID NO: 106),

and further 1-deoxy-D-xylose-5-phosphate reductoisomerase genes from other organisms with the following accession numbers:

AF148852, AY084775, AY054682, AY050802, AY045634, AY081453, AY091405, AY098952, AJ242588, AB009053, AY202991, NP_201085.1, T52570, AF331705_1, BAB16915.1, AF367205_1, AF250235_1, CAC03581.1, CAD22156.1, AF182287_1, DXR_MENPI, ZP_00071219.1, NP_488391.1, ZP_00111307.1, DXR_SYNLE, AAP56260.1, NP_681831.1, NP_442113.1, ZP_00115071.1, ZP_00105106.1, ZP_00113484.1, NP_833540.1, NP_657789.1, NP_661031.1, DXR_BACHD, NP_833080.1, NP_845693.1, NP_562610.1, NP_623020.1, NP_810915.1, NP_243287.1, ZP_00118743.1, NP_464842.1, NP_470690.1, ZP_00082201.1, NP_781898.1, ZP_00123667.1, NP_348420.1, NP_604221.1, ZP_00053349.1, ZP_00064941.1, NP_246927.1, NP_389537.1, ZP_00102576.1, NP_519531.1, AF124757_19, DXR_ZYMMO, NP_713472.1,

NP_459225.1, NP_454827.1, ZP_00045738.1, NP_743754.1, DXR_PSEPK, ZP_00130352.1, NP_702530.1, NP_841744.1, NP_438967.1, AF514841_1, NP_706118.1, ZP_00125845.1, NP_404661.1, NP_285867.1, NP_240064.1, NP_414715.1, ZP_00094058.1, NP_791365.1, ZP_00012448.1, ZP_00015132.1, ZP_00091545.1, NP_629822.1, NP_771495.1, NP_798691.1, NP_231885.1, NP_252340.1, ZP_00022353.1, NP_355549.1, NP_420724.1, ZP_00085169.1, EAA17616.1, NP_273242.1, NP_219574.1, NP_387094.1, NP_296721.1, ZP_00004209.1, NP_823739.1, NP_282934.1, BAA77848.1, NP_660577.1, NP_760741.1, NP_641750.1, NP_636741.1, NP_829309.1, NP_298338.1, NP_444964.1, NP_717246.1, NP_224545.1, ZP_00038451.1, DXR_KITGR, NP_778563.1.

Examples of isopentenyl-diphosphate Δ-isomerase genes are:

a nucleic acid encoding an isopentenyl-diphosphate Δ-isomerase from Adonis palaestina clone ApIPI28, (ipiAa1), ACCESSION #AF188060, published by Cunningham, F.X. Jr. and Gantt, E.: Identification of multi-gene families encoding isopentenyl diphosphate isomerase in plants by heterologous complementation in Escherichia coli, Plant Cell Physiol. 41 (1), 119-123 (2000) (nucleic acid: SEQ ID NO: 107, protein: SEQ ID NO: 108),

and further isopentenyl-diphosphate Δ-isomerase genes from other organisms with the following accession numbers:

Q38929, O48964, Q39472, Q13907, O35586, P58044, O42641, O35760, Q10132, P15496, Q9YB30, Q8YNH4, Q42553, O27997, P50740, O51627, O48965, Q8KFR5, Q39471, Q39664, Q9RVE2, Q01335, Q9HHE4, Q9BXS1, Q9KWF6, Q9CIF5, Q88WB6, Q92BX2, Q8Y7A5, Q8TT35 Q9KK75, Q8NN99, Q8XD58, Q8FE75, Q46822, Q9HP40, P72002, P26173, Q9Z5D3, Q8Z3X9, Q8ZM82, Q9X7Q6, O13504, Q9HFW8, Q8NJL9, Q9UUQ1, Q9NH02, Q9M6K9, Q9M6K5, Q9FXR6, O81691, Q9S7C4, Q8S3L8, Q9M592, Q9M6K3, Q9M6K7, Q9FV48, Q9LLB6, Q9AVJ1, Q9AVG8, Q9M6K6, Q9AVJ5, Q9M6K2, Q9AYS5, Q9M6K8, Q9AVG7, Q8S3L7, Q8W250, Q94IE1, Q9AVI8, Q9AYS6, Q9SAY0, Q9M6K4, Q8GVZ0, Q84RZ8, Q8KZ12, Q8KZ66, Q8FND7, Q88QC9, Q8BFZ6, BAC26382, CAD94476.

Examples of geranyl-diphosphate synthase genes are:

a nucleic acid encoding a geranyl-diphosphate synthase from Arabidopsis thaliana, ACCESSION #Y17376, Bouvier, F., Suire, C., d'Harlingue, A., Backhaus, R.A. and Camara, B.; Molecular cloning of geranyl diphosphate synthase and compartmentation of monoterpene synthesis in plants cells, Plant J. 24 (2), 241-252 (2000) (nucleic acid: SEQ ID NO: 109, protein: SEQ ID NO: 110),

and further geranyl-diphosphate synthase genes from other organisms with the following accession numbers:

Q9FT89, Q8LKJ2, Q9FSW8, Q8LKJ3, Q9SBR3, Q9SBR4, Q9FET8, Q8LKJ1, Q84LG1, Q9JK86

Examples of farnesyl-diphosphate synthase genes are:

a nucleic acid encoding a farnesyl-diphosphate synthase (FPS1) from Arabidopsis thaliana, ACCESSION #U80605, published by Cunillera, N., Arro, M., Delourme, D., Karst, F., Boronat, A. and Ferrer, A.: Arabidopsis thaliana contains two differentially expressed farnesyl-diphosphate synthase genes, J. Biol. Chem. 271 (13), 7774-7780 (1996), (nucleic acid: SEQ ID NO: 111, protein: SEQ ID NO:112),

and further farnesyl-diphosphate synthase genes from other organisms with the following accession numbers:

P53799, P37268, Q02769, Q09152, P49351, O24241, Q43315, P49352, O24242, P49350, P08836, P14324, P49349, P08524, O66952, Q08291, P54383, Q45220, P57537, Q8K9A0, P22939, P45204, O66126, P55539, Q9SWH9, Q9AVI7, Q9FRX2, Q9AYS7, Q94IE8, Q9FXR9, Q9ZWF6, Q9FXR8, Q9AR37, O50009, Q94IE9, Q8RVK7, Q8RVQ7, O04882, Q93RA8, Q93RB0, Q93RB4, Q93RB5, Q93RB3, Q93RB1, Q93RB2, Q920E5.

Examples of geranylgeranyl-diphosphate synthase genes are:

a nucleic acid encoding a geranylgeranyl-diphosphate synthase from Sinapis alba, ACCESSION #X98795, published by Bonk, M., Hoffmann, B., Von Lintig, J., Schledz, M., Al-Babili, S., Hobeika, E., Kleinig, H. and Beyer, P.: Chloroplast import of four carotenoid biosynthetic enzymes in vitro reveals differential fates prior to membrane binding and oligomeric assembly, Eur. J. Biochem. 247 (3), 942-950 (1997), (nucleic acid: SEQ ID NO: 113, protein: SEQ ID NO:114),

and further geranylgeranyl-diphosphate synthase genes from other organisms with the following accession numbers:

P22873, P34802 ,P56966, P80042, Q42698, Q92236, O95749, Q9WTN0, Q50727, P24322, P39464, Q9FXR3, Q9AYN2, Q9FXR2, Q9AVG6, Q9FRW4, Q9SXZ5, Q9AVJ7, Q9AYN1, Q9AVJ4, Q9FXR7, Q8LSC5, Q9AVJ6, Q8LSC4, Q9AVJ3, Q9SSU0, Q9SXZ6, Q9SST9, Q9AVJ0, Q9AVI9, Q9FRW3, Q9FXR5, Q94IF0, Q9FRX1, Q9K567, Q93RA9, Q93QX8, CAD95619, EAA31459

Examples of phytoene synthase genes are:

a nucleic acid encoding a phytoene synthase from Erwinia uredovora, ACCESSION # D90087; published by Misawa, N., Nakagawa, M., Kobayashi, K., Yamano, S., Izawa, Y., Nakamura, K. and Harashima, K.: Elucidation of the Erwinia uredovora carotenoid biosynthetic pathway by functional analysis of gene products expressed in Escherichia coli; J. Bacteriol. 172 (12), 6704-6712 (1990), (nucleic acid: SEQ ID NO: 115, protein: SEQ ID NO: 116),

and further phytoene synthase genes from other organisms with the following accession numbers:

CAB39693, BAC69364, AAF10440, CAA45350, BAA20384, AAM72615, BAC09112, CAA48922, P_001091, CAB84588, AAF41518, CAA48155, AAD38051, AAF33237, AAG10427, AAA34187, BAB73532, CAC19567, AAM62787, CAA55391, AAB65697, AAM45379, CAC27383, AAA32836, AAK07735, BAA84763, P_000205, AAB60314, P_001163, P_000718, AAB71428, AAA34153, AAK07734, CAA42969, CAD76176, CAA68575, P_000130, P_001142, CAA47625, CAA85775, BAC14416, CAA79957, BAC76563, P_000242, P_000551, AAL02001, AAK15621, CAB94795, AAA91951, P_000448

Examples of phytoene desaturase genes are:

a nucleic acid encoding a phytoene desaturase from Erwinia uredovora, ACCESSION # D90087; published by Misawa, N., Nakagawa, M., Kobayashi, K., Yamano, S., Izawa, Y., Nakamura, K. and Harashima, K.: Elucidation of the Erwinia uredovora carotenoid biosynthetic pathway by functional analysis of gene products expressed in Escherichia coli; J. Bacteriol. 172 (12), 6704-6712 (1990), (nucleic acid: SEQ ID NO: 117, protein: SEQ ID NO: 118),

and further phytoene desaturase genes from other organisms with the following accession numbers:

AAL15300, A39597, CAA42573, AAK51545, BAB08179, CAA48195, BAB82461, AAK92625, CAA55392, AAG10426, AAD02489, AAO24235, AAC12846, AAA99519, AAL38046, CAA60479, CAA75094, ZP_001041, ZP_001163, CAA39004, CAA44452, ZP_001142, ZP_000718, BAB82462, AAM45380, CAB56040, ZP_001091, BAC09113, AAP79175, AAL80005, AAM72642, AAM72043, ZP_000745, ZP_001141, BAC07889, CAD55814, ZP_001041, CAD27442, CAE00192, ZP_001163, ZP_000197, BAA18400, AAG10425, ZP_001119, AAF13698, 2121278A, AAB35386, AAD02462, BAB68552, CAC85667, AAK51557, CAA12062, AAG51402, AAM63349,

AAF85796, BAB74081, AAA91161, CAB56041, AAC48983, AAG14399, CAB65434, BAB73487, ZP_001117, ZP_000448, CAB39695, CAD76175, BAC69363, BAA17934, ZP_000171, AAF65586, ZP_000748, BAC07074, ZP_001133, CAA64853, BAB74484, ZP_001156, AAF23289, AAG28703, AAP09348, AAM71569, BAB69140, ZP_000130, AAF41516, AAG18866, CAD95940, NP_656310, AAG10645, ZP_000276, ZP_000192, ZP_000186, AAM94364, EAA31371, ZP_000612, BAC75676, AAF65582

Examples of zeta-carotene desaturase genes are:

a nucleic acid encoding a zeta-carotene desaturase from Narcissus pseudonarcissus, ACCESSION #AJ224683, published by Al-Babili, S., Oelschlegel, J. and Beyer, P.: A cDNA encoding for beta carotene desaturase (Accession No.AJ224683) from Narcissus pseudonarcissus L.. (PGR98-103), Plant Physiol. 117, 719-719 (1998), (nucleic acid: SEQ ID NO: 119, protein: SEQ ID NO: 120),

and further zeta-carotene desaturase genes from other organisms with the following accession numbers:

Q9R6X4, Q38893, Q9SMJ3, Q9SE20, Q9ZTP4, O49901, P74306, Q9FV46, Q9RCT2, ZDS_NARPS, BAB68552.1, CAC85667.1, AF372617_1, ZDS_TARER, CAD55814.1, CAD27442.1, 2121278A, ZDS_CAPAN, ZDS_LYCES, NP_187138.1, AAM63349.1, ZDS_ARATH, AAA91161.1, ZDS_MAIZE, AAG14399.1, NP_441720.1, NP_486422.1, ZP_00111920.1, CAB56041.1, ZP_00074512.1, ZP_00116357.1, NP_681127.1, ZP_00114185.1, ZP_00104126.1, CAB65434.1, NP_662300.1

Examples of crtISO genes are:

a nucleic acid encoding a crtISO from Lycopersicon esculentum; ACCESSION #AF416727, published by Isaacson, T., Ronen, G., Zamir, D. and Hirschberg, J.: Cloning of tangerine from tomato reveals a carotenoid isomerase essential for the production of beta-carotene and xanthophylls in plants; Plant Cell 14 (2), 333-342 (2002), (nucleic acid: SEQ ID NO: 121, protein: SEQ ID NO: 122),

and further crtISO genes from other organisms with the following accession numbers:

AAM53952

Examples of FtsZ genes are:

a nucleic acid encoding an FtsZ from Tagetes erecta, ACCESSION #AF251346, published by Moehs, C.P., Tian, L., Osteryoung, K.W. and Dellapenna, D.: Analysis of carotenoid biosynthetic gene expression during marigold petal development Plant Mol. Biol. 45 (3), 281-293 (2001), (nucleic acid: SEQ ID NO: 123, protein: SEQ ID NO: 124),

and further FtsZ genes from other organisms with the following accession numbers:

CAB89286.1, AF205858_1, NP_200339.1, CAB89287.1, CAB41987.1, AAA82068.1, T06774,AF383876_1, BAC57986.1, CAD22047.1, BAB91150.1, ZP_00072546.1, NP_440816.1, T51092, NP_683172.1, BAA85116.1, NP_487898.1, JC4289, BAA82871.1, NP_781763.1, BAC57987.1, ZP_00111461.1, T51088, NP_190843.1, ZP_00060035.1, NP_846285.1, AAL07180.1, NP_243424.1, NP_833626.1, AAN04561.1, AAN04557.1, CAD22048.1, T51089, NP_692394.1, NP_623237.1, NP_565839.1, T51090, CAA07676.1, NP_113397.1, T51087, CAC44257.1, E84778, ZP_00105267.1, BAA82091.1, ZP_00112790.1, BAA96782.1, NP_348319.1, NP_471472.1, ZP_00115870.1, NP_465556.1, NP_389412.1, BAA82090.1, NP_562681.1, AAM22891.1, NP_371710.1, NP_764416.1, CAB95028.1, FTSZ_STRGR, AF120117_1, NP_827300.1, JE0282, NP_626341.1, AAC45639.1, NP_785689.1, NP_336679.1, NP_738660.1, ZP_00057764.1, AAC32265.1, NP_814733.1, FTSZ_MYCKA, NP_216666.1, CAA75616.1, NP_301700.1, NP_601357.1, ZP_00046269.1, CAA70158.1, ZP_00037834.1, NP_268026.1, FTSZ_ENTHR, NP_787643.1, NP_346105.1, AAC32264.1, JC5548, AAC95440.1, NP_710793.1, NP_687509.1, NP_269594.1, AAC32266.1, NP_720988.1, NP_657875.1, ZP_00094865.1, ZP_00080499.1, ZP_00043589.1, JC7087, NP_660559.1, AAC46069.1, AF179611_14, AAC44223.1, NP_404201.1.

Examples of MinD genes are:

a nucleic acid encoding a MinD from Tagetes erecta, ACCESSION #AF251019, published by Moehs, C.P., Tian, L., Osteryoung, K.W. and Dellapenna, D.: Analysis of carotenoid biosynthetic gene expression during marigold petal development; Plant Mol. Biol. 45 (3), 281-293 (2001), (nucleic acid: SEQ ID NO: 125, protein: SEQ ID NO: 126),

and further MinD genes with the following accession numbers:

NP_197790.1, BAA90628.1, NP_038435.1, NP_045875.1, AAN33031.1, NP_050910.1, CAB53105.1, NP_050687.1, NP_682807.1, NP_487496.1, ZP_00111708.1, ZP_00071109.1, NP_442592.1, NP_603083.1, NP_782631.1, ZP_00097367.1, ZP_00104319.1, NP_294476.1, NP_622555.1, NP_563054.1, NP_347881.1, ZP_00113908.1,

NP_834154.1, NP_658480.1, ZP_00059858.1, NP_470915.1, NP_243893.1, NP_465069.1, ZP_00116155.1, NP_390677.1, NP_692970.1, NP_298610.1, NP_207129.1, ZP_00038874.1, NP_778791.1, NP_223033.1, NP_641561.1, NP_636499.1, ZP_00088714.1, NP_213595.1, NP_743889.1, NP_231594.1, ZP_00085067.1, NP_797252.1, ZP_00136593.1, NP_251934.1, NP_405629.1, NP_759144.1, ZP_00102939.1, NP_793645.1, NP_699517.1, NP_460771.1, NP_860754.1, NP_456322.1, NP_718163.1, NP_229666.1, NP_357356.1, NP_541904.1, NP_287414.1, NP_660660.1, ZP_00128273.1, NP_103411.1, NP_785789.1, NP_715361.1, AF149810_1, NP_841854.1, NP_437893.1, ZP_00022726.1, EAA24844.1, ZP_00029547.1, NP_521484.1, NP_240148.1, NP_770852.1, AF345908_2, NP_777923.1, ZP_00048879.1, NP_579340.1, NP_143455.1, NP_126254.1, NP_142573.1, NP_613505.1, NP_127112.1, NP_712786.1, NP_578214.1, NP_069530.1, NP_247526.1, AAA85593.1, NP_212403.1, NP_782258.1, ZP_00058694.1, NP_247137.1, NP_219149.1, NP_276946.1, NP_614522.1, ZP_00019288.1, CAD78330.1

Nucleic acids which are preferably used as HMG-CoA reductase genes in the above-described preferred embodiment are nucleic acids which encode proteins comprising the amino acid sequence SEQ ID NO: 100, or a sequence derived from this sequence by substitution, insertion or deletion of amino acids which has at least 30%, by preference at least 50%, more preferably at least 70%, even more preferably at least 90%, most preferably at least 95% identity at the amino acid level with the sequence SEQ ID NO: 100 and which has the enzymatic property of an HMG-CoA reductase.

Further examples of HMG-CoA reductases and HMG-CoA reductase genes can be found readily for example from different organisms whose genomic sequence is known, as described above, by homology comparisons of the amino acid sequences or of the corresponding backtranslated nucleic acid sequences from databases with the SEQ ID NO: 100.

Moreover, further examples of HMG-CoA reductases and HMG-CoA reductase genes can be found readily in the manner known per se by hybridization and PCR techniques from different organisms whose genomic sequence is not known, as described above, for example starting from the sequence SEQ ID NO: 99.

In a further especially preferred embodiment nucleic acids are introduced, into organisms, which encode proteins comprising the amino acid sequence of the HMG-CoA reductase of the sequence

SEQ ID NO: 100 in order to increase the HMG-CoA reductase activity.

Suitable nucleic acid sequences can be obtained for example by backtranslating the polypeptide sequence in accordance with the genetic code.

Codons which are preferably used for this purpose are those which are used frequently in accordance with the plant-specific codon usage. The codon usage can be determined readily with the aid of computer evaluations of other, known genes of the organisms in question.

In an especially preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 99 is introduced into the organism.

Nucleic acids which are preferably used as (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase genes in the above-described preferred embodiment are nucleic acids which encode proteins comprising the amino acid sequence SEQ ID NO: 102, or a sequence derived from this sequence by substitution, insertion or deletion of amino acids which has at least 30%, by preference at least 50%, more preferably at least 70%, even more preferably at least 90%, most preferably at least 95% identity at the amino acid level with the sequence SEQ ID NO: 102 and which has the enzymatic property of an (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase.

Further examples of (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductases and (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase genes can be found readily for example from different organisms whose genomic sequence is known, as described above, by homology comparisons of the amino acid sequences or of the corresponding backtranslated nucleic acid sequences from databases with the SEQ ID NO: 102.

Moreover, further examples of (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductases and (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase genes can be found readily in the manner known per se by hybridization and PCR techniques from different organisms whose genomic sequence is not known, as described above, for example starting from the sequence SEQ ID NO: 101.

In a further especially preferred embodiment nucleic acids are introduced, into organisms, which encode proteins comprising the amino acid sequence of the (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase of the sequence SEQ ID NO: 102 in order to

increase the (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity.

Suitable nucleic acid sequences can be obtained for example by backtranslating the polypeptide sequence in accordance with the genetic code.

Codons which are preferably used for this purpose are those which are used frequently in accordance with the plant-specific codon usage. The codon usage can be determined readily with the aid of computer evaluations of other, known genes of the organisms in question.

In an especially preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 101 is introduced into the organism.

Nucleic acids which are preferably used as 1-deoxy-D-xylose-5-phosphate synthase genes in the above-described preferred embodiment are nucleic acids which encode proteins comprising the amino acid sequence SEQ ID NO: 104, or a sequence derived from this sequence by substitution, insertion or deletion of amino acids which has at least 30%, by preference at least 50%, more preferably at least 70%, even more preferably at least 90%, most preferably at least 95% identity at the amino acid level with the sequence SEQ ID NO: 104 and which has the enzymatic property of a 1-deoxy-D-xylose-5-phosphate synthase.

Further examples of 1-deoxy-D-xylose-5-phosphate synthases and 1-deoxy-D-xylose-5-phosphate synthase genes can be found readily for example from different organisms whose genomic sequence is known, as described above, by homology comparisons of the amino acid sequences or of the corresponding backtranslated nucleic acid sequences from databases with the SEQ ID NO: 104.

Moreover, further examples of 1-deoxy-D-xylose-5-phosphate synthases and 1-deoxy-D-xylose-5-phosphate synthase genes can be found readily in the manner known per se by hybridization and PCR techniques from different organisms whose genomic sequence is not known, as described above, for example starting from the sequence SEQ ID NO: 103.

In a further especially preferred embodiment nucleic acids are introduced, into organisms, which encode proteins comprising the amino acid sequence of the 1-deoxy-D-xylose-5-phosphate synthase of the sequence SEQ ID NO: 104 in order to increase the 1-deoxy-D-xylose-5-phosphate synthase activity.

Suitable nucleic acid sequences can be obtained for example by backtranslating the polypeptide sequence in accordance with the genetic code.

Codons which are preferably used for this purpose are those which are used frequently in accordance with the plant-specific codon usage. The codon usage can be determined readily with the aid of computer evaluations of other, known genes of the organisms in question.

In an especially preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 103 is introduced into the organism.

Nucleic acids which are preferably used as 1-deoxy-D-xylose-5-phosphate reductoisomerase genes in the above-described preferred embodiment are nucleic acids which encode proteins comprising the amino acid sequence SEQ ID NO: 106, or a sequence derived from this sequence by substitution, insertion or deletion of amino acids which has at least 30%, by preference at least 50%, more preferably at least 70%, even more preferably at least 90%, most preferably at least 95% identity at the amino acid level with the sequence SEQ ID NO: 106 and which has the enzymatic property of a 1-deoxy-D-xylose-5-phosphate reductoisomerase.

Further examples of 1-deoxy-D-xylose-5-phosphate reductoisomerases and 1-deoxy-D-xylose-5-phosphate reductoisomerase genes can be found readily for example from different organisms whose genomic sequence is known, as described above, by homology comparisons of the amino acid sequences or of the corresponding backtranslated nucleic acid sequences from databases with the SEQ ID NO: 106.

Moreover, further examples of 1-deoxy-D-xylose-5-phosphate reductoisomerases and 1-deoxy-D-xylose-5-phosphate reductoisomerase genes can be found readily in the manner known per se by hybridization and PCR techniques from different organisms whose genomic sequence is not known, as described above, for example starting from the sequence SEQ ID NO: 105.

In a further especially preferred embodiment nucleic acids are introduced, into organisms, which encode proteins comprising the amino acid sequence of the 1-deoxy-D-xylose-5-phosphate reductoisomerase of the sequence SEQ ID NO: 106 in order to increase the 1-deoxy-D-xylose-5-phosphate reductoisomerase activity.

Suitable nucleic acid sequences can be obtained for example by backtranslating the polypeptide sequence in accordance with the genetic code.

Codons which are preferably used for this purpose are those which are used frequently in accordance with the plant-specific codon usage. The codon usage can be determined readily with the aid of computer evaluations of other, known genes of the organisms in question.

In an especially preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 105 is introduced into the organism.

Nucleic acids which are preferably used as isopentenyl-diphosphate Δ-isomerase genes in the above-described preferred embodiment are nucleic acids which encode proteins comprising the amino acid sequence SEQ ID NO: 108, or a sequence derived from this sequence by substitution, insertion or deletion of amino acids which has at least 30%, by preference at least 50%, more preferably at least 70%, even more preferably at least 90%, most preferably at least 95% identity at the amino acid level with the sequence SEQ ID NO: 108 and which has the enzymatic property of an isopentenyl-diphosphate Δ-isomerase.

Further examples of isopentenyl-diphosphate Δ-isomerases and isopentenyl-diphosphate Δ-isomerase genes can be found readily for example from different organisms whose genomic sequence is known, as described above, by homology comparisons of the amino acid sequences or of the corresponding backtranslated nucleic acid sequences from databases with the SEQ ID NO: 108.

Moreover, further examples of isopentenyl-diphosphate Δ-isomerases and isopentenyl-diphosphate Δ-isomerase genes can be found readily in the manner known per se by hybridization and PCR techniques from different organisms whose genomic sequence is not known, as described above, for example starting from the sequence SEQ ID NO: 107.

In a further especially preferred embodiment nucleic acids are introduced, into organisms, which encode proteins comprising the amino acid sequence of the isopentenyl-diphosphate Δ-isomerase of the sequence SEQ ID NO: 108 in order to increase the isopentenyl-diphosphate Δ-isomerase activity.

Suitable nucleic acid sequences can be obtained for example by backtranslating the polypeptide sequence in accordance with the genetic code.

Codons which are preferably used for this purpose are those which are used frequently in accordance with the plant-specific codon usage. The codon usage can be determined readily with the aid of computer evaluations of other, known genes of the organisms in question.

In an especially preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 107 is introduced into the organism.

Nucleic acids which are preferably used as geranyl-diphosphate synthase genes in the above-described preferred embodiment are nucleic acids which encode proteins comprising the amino acid sequence SEQ ID NO: 110, or a sequence derived from this sequence by substitution, insertion or deletion of amino acids which has at least 30%, by preference at least 50%, more preferably at least 70%, even more preferably at least 90%, most preferably at least 95% identity at the amino acid level with the sequence SEQ ID NO: 110 and which has the enzymatic property of a geranyl-diphosphate synthase

Further examples of geranyl-diphosphate synthases and geranyl-diphosphate synthase genes can be found readily for example from different organisms whose genomic sequence is known, as described above, by homology comparisons of the amino acid sequences or of the corresponding backtranslated nucleic acid sequences from databases with the SEQ ID NO: 110.

Moreover, further examples of geranyl-diphosphate synthases and geranyl-diphosphate synthase genes can be found readily in the manner known per se by hybridization and PCR techniques from different organisms whose genomic sequence is not known, as described above, for example starting from the sequence SEQ ID NO: 109.

In a further especially preferred embodiment nucleic acids are introduced, into organisms, which encode proteins comprising the amino acid sequence of the geranyl-diphosphate synthase of the sequence SEQ ID NO: 110 in order to increase the geranyl-diphosphate synthase activity.

Suitable nucleic acid sequences can be obtained for example by backtranslating the polypeptide sequence in accordance with the genetic code.

Codons which are preferably used for this purpose are those which are used frequently in accordance with the plant-specific codon usage. The codon usage can be determined readily with the aid of computer evaluations of other, known genes of the organisms in question.

In an especially preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 109 is introduced into the organism.

Nucleic acids which are preferably used as farnesyl-diphosphate synthase genes in the above-described preferred embodiment are nucleic acids which encode proteins comprising the amino acid sequence SEQ ID NO: 112, or a sequence derived from this sequence by substitution, insertion or deletion of amino acids which has at least 30%, by preference at least 50%, more preferably at least 70%, even more preferably at least 90%, most preferably at least 95% identity at the amino acid level with the sequence SEQ ID NO: 112 and which has the enzymatic property of a farnesyl-diphosphate synthase.

Further examples of farnesyl-diphosphate synthases and farnesyl-diphosphate synthase genes can be found readily for example from different organisms whose genomic sequence is known, as described above, by homology comparisons of the amino acid sequences or of the corresponding backtranslated nucleic acid sequences from databases with the SEQ ID NO: 112.

Moreover, further examples of farnesyl-diphosphate synthases and farnesyl-diphosphate synthase genes can be found readily in the manner known per se by hybridization and PCR techniques from different organisms whose genomic sequence is not known, as described above, for example starting from the sequence SEQ ID NO: 111.

In a further especially preferred embodiment nucleic acids are introduced, into organisms, which encode proteins comprising the amino acid sequence of the farnesyl-diphosphate synthase of the sequence SEQ ID NO: 112 in order to increase the farnesyl-diphosphate synthase activity.

Suitable nucleic acid sequences can be obtained for example by backtranslating the polypeptide sequence in accordance with the genetic code.

Codons which are preferably used for this purpose are those which are used frequently in accordance with the plant-specific codon usage. The codon usage can be determined readily with the aid of computer evaluations of other, known genes of the organisms in question.

In an especially preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 111 is introduced into the organism.

Nucleic acids which are preferably used as geranylgeranyl-diphosphate synthase genes in the above-described preferred embodiment are nucleic acids which encode proteins comprising the amino acid sequence SEQ ID NO: 114, or a sequence derived from this sequence by substitution, insertion or deletion of amino acids which has at least 30%, by preference at least 50%, more preferably at least 70%, even more preferably at least 90%, most preferably at least 95% identity at the amino acid level with the sequence SEQ ID NO: 114 and which has the enzymatic property of a geranylgeranyl-diphosphate synthase.

Further examples of geranylgeranyl-diphosphate synthases and geranylgeranyl-diphosphate synthase genes can be found readily for example from different organisms whose genomic sequence is known, as described above, by homology comparisons of the amino acid sequences or of the corresponding backtranslated nucleic acid sequences from databases with the SEQ ID NO: 114.

Moreover, further examples of geranylgeranyl-diphosphate synthases and geranylgeranyl-diphosphate synthase genes can be found readily in the manner known per se by hybridization and PCR techniques from different organisms whose genomic sequence is not known, as described above, for example starting from the sequence SEQ ID NO: 113.

In a further especially preferred embodiment nucleic acids are introduced, into organisms, which encode proteins comprising the amino acid sequence of the geranylgeranyl-diphosphate synthase of the sequence SEQ ID NO: 114 in order to increase the geranylgeranyl-diphosphate synthase activity.

Suitable nucleic acid sequences can be obtained for example by backtranslating the polypeptide sequence in accordance with the genetic code.

Codons which are preferably used for this purpose are those which are used frequently in accordance with the plant-specific codon usage. The codon usage can be determined readily with the aid of computer evaluations of other, known genes of the organisms in question.

In an especially preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 113 is introduced into the organism.

Nucleic acids which are preferably used as phytoene synthase genes in the above-described preferred embodiment are nucleic acids which encode proteins comprising the amino acid sequence SEQ ID NO: 116, or a sequence derived from this sequence by substitution, insertion or deletion of amino acids which has at least 30%, by preference at least 50%, more preferably at least 70%, even more preferably at least 90%, most preferably at least 95% identity at the amino acid level with the sequence SEQ ID NO: 116 and which has the enzymatic property of a phytoene synthase.

Further examples of phytoene synthases and phytoene synthase genes can be found readily for example from different organisms whose genomic sequence is known, as described above, by homology comparisons of the amino acid sequences or of the corresponding backtranslated nucleic acid sequences from databases with the SEQ ID NO: 116.

Moreover, further examples of phytoene synthases and phytoene synthase genes can be found readily in the manner known per se by hybridization and PCR techniques from different organisms whose genomic sequence is not known, as described above, for example starting from the sequence SEQ ID NO: 115.

In a further especially preferred embodiment nucleic acids are introduced, into organisms, which encode proteins comprising the amino acid sequence of the phytoene synthase of the sequence SEQ ID NO: 116 in order to increase the phytoene synthase activity.

Suitable nucleic acid sequences can be obtained for example by backtranslating the polypeptide sequence in accordance with the genetic code.

Codons which are preferably used for this purpose are those which are used frequently in accordance with the plant-specific codon usage. The codon usage can be determined readily with the aid of computer evaluations of other, known genes of the organisms in question.

In an especially preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 115 is introduced into the organism.

Nucleic acids which are preferably used as phytoene desaturase genes in the above-described preferred embodiment are nucleic acids which encode proteins comprising the amino acid sequence SEQ ID NO: 118, or a sequence derived from this sequence by substitution, insertion or deletion of amino acids which has at least 30%, by preference at least 50%, more preferably at least 70%, even more preferably at least 90%, most preferably at least 95% identity at the amino acid level with the sequence SEQ ID NO: 118 and which has the enzymatic property of a phytoene desaturase.

Further examples of phytoene desaturases and phytoene desaturase genes can be found readily for example from different organisms whose genomic sequence is known, as described above, by homology comparisons of the amino acid sequences or of the corresponding backtranslated nucleic acid sequences from databases with the SEQ ID NO: 118.

Moreover, further examples of phytoene desaturases and phytoene desaturase genes can be found readily in the manner known per se by hybridization and PCR techniques from different organisms whose genomic sequence is not known, as described above, for example starting from the sequence SEQ ID NO: 117.

In a further especially preferred embodiment nucleic acids are introduced, into organisms, which encode proteins comprising the amino acid sequence of the phytoene desaturase of the sequence SEQ ID NO: 118 in order to increase the phytoene desaturase activity.

Suitable nucleic acid sequences can be obtained for example by backtranslating the polypeptide sequence in accordance with the genetic code.

Codons which are preferably used for this purpose are those which are used frequently in accordance with the plant-specific codon usage. The codon usage can be determined readily with the aid of

computer evaluations of other, known genes of the organisms in question.

In an especially preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 117 is introduced into the organism.

Nucleic acids which are preferably used as zeta-carotene desaturase genes in the above-described preferred embodiment are nucleic acids which encode proteins comprising the amino acid sequence SEQ ID NO: 120, or a sequence derived from this sequence by substitution, insertion or deletion of amino acids which has at least 30%, by preference at least 50%, more preferably at least 70%, even more preferably at least 90%, most preferably at least 95% identity at the amino acid level with the sequence SEQ ID NO: 120 and which has the enzymatic property of a zeta-carotene desaturase.

Further examples of zeta-carotene desaturases and zeta-carotene desaturase genes can be found readily for example from different organisms whose genomic sequence is known, as described above, by homology comparisons of the amino acid sequences or of the corresponding backtranslated nucleic acid sequences from databases with the SEQ ID NO: 120.

Moreover, further examples of zeta-carotene desaturases and zeta-carotene desaturase genes can be found readily in the manner known per se by hybridization and PCR techniques from different organisms whose genomic sequence is not known, as described above, for example starting from the sequence SEQ ID NO: 119.

In a further especially preferred embodiment nucleic acids are introduced, into organisms, which encode proteins comprising the amino acid sequence of the zeta-carotene desaturase of the sequence SEQ ID NO: 120 in order to increase the zeta-carotene desaturase activity.

Suitable nucleic acid sequences can be obtained for example by backtranslating the polypeptide sequence in accordance with the genetic code.

Codons which are preferably used for this purpose are those which are used frequently in accordance with the plant-specific codon usage. The codon usage can be determined readily with the aid of computer evaluations of other, known genes of the organisms in question.

In an especially preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 119 is introduced into the organism.

Nucleic acids which are preferably used as CrtISO genes in the above-described preferred embodiment are nucleic acids which encode proteins comprising the amino acid sequence SEQ ID NO: 122, or a sequence derived from this sequence by substitution, insertion or deletion of amino acids which has at least 30%, by preference at least 50%, more preferably at least 70%, even more preferably at least 90%, most preferably at least 95% identity at the amino acid level with the sequence SEQ ID NO: 122 and which has the enzymatic property of a CrtISO.

Further examples of CrtISO and CrtISO genes can be found readily for example from different organisms whose genomic sequence is known, as described above, by homology comparisons of the amino acid sequences or of the corresponding backtranslated nucleic acid sequences from databases with the SEQ ID NO: 122.

Moreover, further examples of CrtISO and CrtISO genes can be found readily in the manner known per se by hybridization and PCR techniques from different organisms whose genomic sequence is not known, as described above, for example starting from the sequence SEQ ID NO: 121.

In a further especially preferred embodiment nucleic acids are introduced, into organisms, which encode proteins comprising the amino acid sequence of the CrtISO of the sequence SEQ ID NO: 122 in order to increase the CrtISO activity.

Suitable nucleic acid sequences can be obtained for example by backtranslating the polypeptide sequence in accordance with the genetic code.

Codons which are preferably used for this purpose are those which are used frequently in accordance with the plant-specific codon usage. The codon usage can be determined readily with the aid of computer evaluations of other, known genes of the organisms in question.

In an especially preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 121 is introduced into the organism.

Nucleic acids which are preferably used as FtsZ genes in the above-described preferred embodiment are nucleic acids which encode proteins comprising the amino acid sequence SEQ ID NO: 124, or a sequence derived from this sequence by

substitution, insertion or deletion of amino acids which has at least 30%, by preference at least 50%, more preferably at least 70%, even more preferably at least 90%, most preferably at least 95% identity at the amino acid level with the sequence SEQ ID NO: 124 and which has the enzymatic property of an FtsZ.

Further examples of FtsZ and FtsZ genes can be found readily for example from different organisms whose genomic sequence is known, as described above, by homology comparisons of the amino acid sequences or of the corresponding backtranslated nucleic acid sequences from databases with the SEQ ID NO: 124.

Moreover, further examples of FtsZ and FtsZ genes can be found readily in the manner known per se by hybridization and PCR techniques from different organisms whose genomic sequence is not known, as described above, for example starting from the sequence SEQ ID NO: 123.

In a further especially preferred embodiment nucleic acids are introduced, into organisms, which encode proteins comprising the amino acid sequence of the FtsZ of the sequence SEQ ID NO: 124 in order to increase the FtsZ activity.

Suitable nucleic acid sequences can be obtained for example by backtranslating the polypeptide sequence in accordance with the genetic code.

Codons which are preferably used for this purpose are those which are used frequently in accordance with the plant-specific codon usage. The codon usage can be determined readily with the aid of computer evaluations of other, known genes of the organisms in question.

In an especially preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 123 is introduced into the organism.

Nucleic acids which are preferably used as MinD genes in the above-described preferred embodiment are nucleic acids which encode proteins comprising the amino acid sequence SEQ ID NO: 126, or a sequence derived from this sequence by substitution, insertion or deletion of amino acids which has at least 30%, by preference at least 50%, more preferably at least 70%, even more preferably at least 90%, most preferably at least 95% identity at the amino acid level with the sequence SEQ ID NO: 126 and which has the enzymatic property of a MinD.

Further examples of MinD and MinD genes can be found readily for example from different organisms whose genomic sequence is known, as described above, by homology comparisons of the amino acid sequences or of the corresponding backtranslated nucleic acid sequences from databases with the SEQ ID NO: 126.

Moreover, further examples of MinD and MinD genes can be found readily in the manner known per se by hybridization and PCR techniques from different organisms whose genomic sequence is not known, as described above, for example starting from the sequence SEQ ID NO: 125.

In a further especially preferred embodiment nucleic acids are introduced, into organisms, which encode proteins comprising the amino acid sequence of the MinD of the sequence SEQ ID NO: 126 in order to increase the MinD activity.

Suitable nucleic acid sequences can be obtained for example by backtranslating the polypeptide sequence in accordance with the genetic code.

Codons which are preferably used for this purpose are those which are used frequently in accordance with the plant-specific codon usage. The codon usage can be determined readily with the aid of computer evaluations of other, known genes of the organisms in question.

In an especially preferred embodiment, a nucleic acid comprising the sequence SEQ ID NO: 125 is introduced into the organism.

All of the abovementioned HMG-CoA reductase genes, (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase genes, 1-deoxy-D-xylose-5-phosphate synthase genes, 1-deoxy-D-xylose-5-phosphate reductoisomerase genes, isopentenyl-diphosphate Δ-isomerase genes, geranyl-diphosphate synthase genes, farnesyl-diphosphate synthase genes, geranylgeranyl-diphosphate synthase genes, phytoene synthase genes, phytoene desaturase genes, zeta-carotene desaturase genes, crtISO genes, FtsZ genes or MinD genes can furthermore be generated in the manner which is known per se by chemical synthesis, starting with the nucleotide units, such as, for example, by fragment condensation of individual overlapping complementary nucleic acid units of the double helix. Oligonucleotides can be synthesized chemically for example in the known manner by the phosphoamidite method (Voet, Voet, 2nd edition, Wiley Press New York, pages 896-897). The annealing of synthetic oligonucleotides, and filling of gaps with the aid of

the Klenow fragment of the DNA polymerase and ligation reactions and general cloning methods are described in Sambrook et al. (1989), Molecular cloning: A laboratory manual, Cold Spring Harbor Laboratory Press.

In a further preferred embodiment of the method, the plants additionally show a reduced endogenous β-hydroxylase activity in comparison with the wild type.

As mentioned above, a reduced activity is preferably understood as meaning the partial or essentially complete prevention or blockage of the functionality of an enzyme in a plant cell, plant or a part, tissue, organ, cells or seeds thereof, as the result of different cell-biological mechanisms.

Reducing an activity in plants in comparison with the wild type can be effected for example by reducing the amount of protein or the amount of mRNA in the plant. Accordingly, an activity which is reduced in comparison with the wild type can be determined directly or can be carried out via the determination of the amount of protein, or the amount of mRNA, of the plant according to the invention in comparison with the wild type.

A reduction of an activity comprises a quantitative reduction of a protein down to an essentially complete absence of the protein (i.e. lacking detectability of the activity in question or lacking immunological detectability of the protein in question).

Endogenous β-hydroxylase activity is understood as meaning the enzyme activity of the endogenous β-hydroxylase which is homologous to the plant.

An endogenous β-hydroxylase is understood as meaning an endogenous hydroxylase which is homologous to the plant, as described above. If, for example, Tagetes erecta is the target plant to be genetically modified, the endogenous β-hydoxylase is understood as meaning the β-hydoxylase of Tagetes erecta.

Accordingly, an endogenous β-hydroxylase is understood as meaning in particular a protein which is homologous to the plant and which has enzymatic activity of converting β-carotene into zeaxanthin.

Accordingly, endogenous β-hydroxylase activity is understood as meaning the amount of β-carotene converted, or the amount of zeaxanthin formed, by the protein endogenous β-hydroxylase within a certain period of time.

Thus, in the case of a reduced endogenous β-hydroxylase activity in comparison with the wild type, the amount of β-carotene converted, or the amount of zeaxanthin formed, by the protein endogenous β-hydroxylase within a certain period of time, is reduced in comparison with the wild type.

By preference, this reduction of the endogenous β-hydroxylase activity amounts to at least 5%, further preferably to at least 20%, further preferably to at least 50%, further preferably to 100%. It is especially preferred that endogenous β-hydroxylase activity is completely eliminated.

Surprisingly, it has been found that, in plants which predominantly produce carotenoids of the α-carotene pathway, such as, for example, lutein, such as, for example, plants of the genus Tagetes, it is advantageous to reduce the activity of the endogenous β-hydroxylase and, if appropriate, to increase the activity of a heterologous hydroxylase. It is especially preferred to use, in this context, hydroxylases or functional equivalents thereof which are derived from plants which predominantly produce carotenoids of the β-carotene pathway, such as, for example, the above-described β-hydroxylase from tomato (nucleic acid: SEQ ID No. 97, protein: SEQ ID No. 98).

The endogenous β-hydroxylase activity is determined as described above analogously to the hydroxylase activity.

Preferably, the reduction of endogenous β-hydroxylase activity in plants is effected by at least one of the following methods:

a) introducing, into plants, at least one double-stranded endogenous β-hydroxylase ribonucleic acid sequence, hereinbelow also referred to as endogenous β-hydroxylase dsRNA, or (an) expression cassette(s) which ensure(s) its expression.

   Comprised are those methods in which the endogenous β-hydroxylase dsRNA is directed against an endogenous β-hydroxylase gene (i.e. genomic DNA sequences, such as the promoter sequence) or an endogenous β-hydroxylase transcript (i.e. mRNA sequences)

b) introducing, into plants, at least one endogenous β-hydroxylase antisense ribonucleic acid sequence, hereinbelow also referred to as endogenous β-hydroxylase antisense RNA, or an expression cassette which ensures its expression. Comprised are those methods in which the endogenous β-hydroxylase antisense RNA is directed against an endogenous β-hydroxylase gene (i.e. genomic DNA sequences) or against an endogenous β-hydroxylase gene transcript (i.e. RNA sequences). Also comprised are α-anomeric nucleic acid sequences,

c) introducing, into plants, at least one endogenous β-hydroxylase antisense RNA in combination with a ribozyme or (an) expression cassette which ensures its expression,

d) introducing, into plants, at least one endogenous β-hydroxylase sense ribonucleic acid sequence, hereinafter also referred to as endogenous β-hydroxylase sense RNA, for inducing a cosuppression or an expression cassette which ensures its expression,

e) introducing, into plants, at least one DNA- or protein-binding factor against an endogenous β-hydroxylase gene, an endogenous β-hydroxylase RNA or an endogenous β-hydroxylase protein or one expression cassette which ensures its expression,

f) introducing, into plants, at least one viral nucleic acid sequence which brings about the degradation of endogenous β-hydroxylase RNA or an expression cassette which ensures its expression,

g) introducing, into plants, at least one construct for generating a loss of function, such as, for example, the generation of stop codons or a reading-frame shift, at an endogenous β-hydroxylase gene, for example by generating an insertion, deletion, inversion or mutation in an endogenous β-hydroxylase gene. Preferably, knock-out mutants can be generated by means of site-specific insertion into said endogenous β-hydroxylase gene by means of homologous recombination or introduction of sequence-specific nucleases against endogenous β-hydroxylase gene sequences.

The skilled worker is familiar with the fact that other methods may also be employed within the scope of the present invention for reducing an endogenous β-hydroxylase or its activity or function. For example, the introduction of a dominant-negative variant of an endogenous β-hydroxylase, or of an expression cassette which ensures its expression, may also be advantageous. Here, each and any of these methods may bring about a reduction of the amount of protein, the amount of mRNA and/or the activity of an endogenous β-hydroxylase. A combined application is also

feasible. Further methods are known to the skilled worker and may comprise the prevention or repression of the processing of endogenous β-hydroxylase, of transport of endogenous β-hydroxylase or its mRNA, inhibition of ribosome attachment, inhibition of RNA splicing, induction of an endogenous β-hydroxylase-RNA-degrading enzyme and/or inhibition of the elongation or termination of the translation.

The individual preferred methods shall be described hereinbelow by examples of embodiments:

a) introducing a double-stranded, endogenous β-hydroxylase ribonucleic acid sequence (endogenous β-hydroxylase dsRNA)

The method of regulating genes by means of double-stranded RNA has been described in detail hereinabove for reducing the ε-cyclase activity. This method can be carried out analogously for reducing the endogenous β-hydroxylase activity.

A double-stranded endogenous β-hydroxylase ribonucleic acid sequence, or else endogenous β-hydroxylase dsRNA, is preferably understood as meaning an RNA molecule which has a region with double-stranded structure and comprises, in this region, a nucleic acid sequence which

a) is identical to at least a part of the plant's homologous endogenous β-hydroxylase transcript and/or

b) is identical to at least a part of the plant's homologous endogenous β-hydroxylase promoter sequence.

Thus, it is preferred, in the method according to the invention, to introduce into plants, in order to reduce the endogenous β-hydroxylase activity, an RNA which has a region with double-stranded structure and comprises, in this region, a nucleic acid sequence which

a) is identical to at least a part of the plant's homologous endogenous β-hydroxylase transcript and/or

b) is identical to at least a part of the plant's homologous endogenous β-hydroxylase promoter sequence.

The term "endogenous β-hydroxylase transcript" is understood as meaning the transcribed part of an endogenous β-hydroxylase gene which, in addition to the endogenous β-hydroxylase-coding

sequence, for example also comprises noncoding sequences such as, for example, UTRs.

An RNA which "is identical to at least a part of the plant's homologous endogenous β-hydroxylase promoter sequence" preferably means that the RNA sequence is identical to at least a part of the theoretical transcript of the endogenous β-hydroxylase promoter sequence, i.e. to the corresponding RNA sequence.

"A part" of the plant's homologous endogenous β-hydroxylase transcript, or the plant's homologous endogenous β-hydroxylase promoter sequence, is understood as meaning part-sequences which may reach from a few base pairs up to complete sequences of the transcript, or of the promoter sequence. The skilled worker can readily determine the optimal length of the part-sequences by routine experimentation.

As a rule, the length of the part-sequences amounts to at least 10 bases and not more than 2 kb, preferably at least 25 bases and not more than 1.5 kb, especially preferably at least 50 bases and not more than 600 bases, very especially preferably at least 100 bases and not more than 500, most preferably at least 200 bases or at least 300 bases and not more than 400 bases.

Preferably, the part-sequences are selected in such a way that as high as possible a specificity is achieved and that it is avoided that activities of other enzymes are reduced whose reduction is not desired. Thus, it is advantageous to select, for the part-sequences of the endogenous β-hydroxylase dsRNA, parts of the endogenous β-hydroxylase transcripts and/or part-sequences of the endogenous β-hydroxylase promoter sequences which are not found in other activities.

Thus, in an especially preferred embodiment, the endogenous β-hydroxylase-dsRNA comprises a sequence which is identical to a part of the plant's homologous endogenous β-hydroxylase transcript and which comprises the 5'-terminus or the 3'-terminus of the plant's homologous nucleic acid encoding an endogenous β-hydroxylase. Untranslated regions 5' or 3' of the transcript are especially suitable for generating selective double-stranded structures.

The invention furthermore relates to double-stranded RNA molecules (dsRNA molecules) which, when introduced into a plant organism (or a cell, tissue, organ or propagation material derived therefrom), bring about the reduction of an endogenous

β-hydroxylase.

Furthermore, the invention relates to a double-stranded RNA molecule for reducing the expression of an endogenous β-hydroxylase (endogenous β-hydroxylase dsRNA), which preferably comprises

a) a sense RNA strand comprising at least one ribonucleotide sequence which is essentially identical to at least a part of a sense RNA endogenous β-hydroxylase transcript, and

b) an antisense RNA strand which is essentially, preferably fully, complementary to the RNA sense strand.

To transform the plant with an endogenous β-hydroxylase dsRNA, it is preferred to use a nucleic acid construct which is introduced into the plant and which is transcribed in the plant into the endogenous β-hydroxylase dsRNA.

Furthermore, the present invention also relates to a nucleic acid construct which can be transcribed into

a) a sense RNA strand comprising at least one ribonucleotide sequence which is essentially identical to at least a part of the sense RNA endogenous β-hydroxylase transcript, and

b) an antisense RNA strand which is essentially, preferably fully, complementary to the RNA sense strand in a).

These nucleic acid constructs are hereinbelow also referred to as expression cassettes or expression vectors.

As regards the dsRNA molecules, endogenous β-hydroxylase nucleic acid sequence, or the corresponding transcript, is preferably understood as meaning the sequence in accordance with SEQ ID NO: 127 or a part of the same.

"Essentially identical" means that the dsRNA sequence may also comprise insertions, deletions and individual point mutations in comparison with the endogenous β-hydroxylase target sequence while still bringing about an efficient reduction of the expression. Preferably, the homology amounts to at least 75%, preferably at least 80%, very especially preferably at least 90%, most preferably 100%, between the sense strand of an inhibitory dsRNA and at least a part of the sense RNA transcript of an endogenous β-hydroxylase gene, or between the antisense strand, the

complementary strand of an endogenous β-hydroxylase gene.

100% sequence identity between dsRNA and an endogenous β-hydroxylase gene transcript is not necessarily required in order to bring about an efficient reduction of the endogenous β-hydroxylase expression. Accordingly, there is the advantage that the method is tolerant to sequence deviations as can be present due to genetic mutations, polymorphisms or evolutionary divergences. Thus, for example, using the dsRNA which has been generated starting from the endogenous β-hydroxylase sequence of the one organism, it is possible to suppress the endogenous β-hydroxylase expression in another organism. To this end, the dsRNA preferably comprises sequence regions of endogenous β-hydroxylase gene transcripts which correspond to conserved regions. Said conserved regions can be deduced readily from sequence comparisons.

As an alternative, an "essentially identical" dsRNA can also be defined as a nucleic acid sequence which is capable of hybridizing with a part of an endogenous β-hydroxylase gene transcript (for example in 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA at 50°C or 70°C for 12 to 16 h).

"Essentially complementary" means that the antisense RNA strand may also show insertions, deletions and individual point mutations in comparison with the complement of the sense RNA strand. Preferably, the homology amounts to at least 80%, preferably at least 90%, very especially preferably at least 95%, most preferably 100%, between the antisense RNA strand and the complement of the sense RNA strand.

In a further embodiment, the endogenous β-hydroxylase dsRNA comprises

a) a sense RNA strand comprising at least one ribonucleotide sequence which is essentially identical to at least a part of the sense RNA transcript of the promoter region of an endogenous β-hydroxylase gene, and

b) an antisense RNA strand which is essentially, preferably fully, complementary to the RNA sense strand.

The corresponding nucleic acid construct which is preferably used for the transformation of the plants comprises

a) a sense DNA strand which is essentially identical to at least a part of the promoter region of an endogenous β-hydroxylase

gene, and

b) an antisense DNA strand which is essentially, preferably fully, complementary to the DNA sense strand in a).

To generate the endogenous β-hydroxylase sequences for reducing the endogenous β-hydroxylase activity, it is especially preferred to use the following part-sequences, in particular for *Tagetes erecta*:

SEQ ID NO: 163: sense fragment of the 5'-terminal region of the endogenous β-hydroxylase

SEQ ID NO: 164: antisense fragment of the 5'-terminal region of the endogenous β-hydroxylase

The dsRNA can consist of one or more strands of polyribonucleotides. To achieve the same purpose, it is, naturally, also possible to introduce, into the cell or the organism, several individual dsRNA molecules, each of which comprises one of the above-defined ribonucleotide sequence segments.

The double-stranded dsRNA structure can be formed starting from two complementary separate RNA strands or – preferably – starting from an individual autocomplementary RNA strand. In this case, sense RNA strand and antisense RNA strand are preferably covalently linked with one another in the form of an inverted repeat.

As described for example in WO 99/53050, the dsRNA may also comprise a hairpin structure by sense and antisense strand being linked by a linking sequence (linker; for example an intron). The autocomplementary dsRNA structures are preferred since they merely require the expression of one RNA sequence and always comprise the complementary RNA strands in an equimolar ratio. The linking sequence is preferably an intron (for example an intron of the potato ST-LS1 gene; Vancanneyt GF et al. (1990) Mol Gen Genet 220(2):245-250).

The nucleic acid sequence encoding a dsRNA may comprise further elements such as, for example, transcription termination or polyadenylation signals.

Further preferred embodiments for reducing the endogenous β-hydroxylase activity result analogously to the above-described, preferred embodiments for reducing the ε-cyclase activity by substituting ε-cyclase for endogenous β-hydroxylase.

Plants which are especially preferably used in the method according to the invention are genetically modified plants with the following combinations of genetic modifications:

genetically modified plants which, in comparison with the wild type, have an increased or generated ketolase activity in petals and an increased hydroxylase activity,

genetically modified plants which, in comparison with the wild type, have an increased or generated ketolase activity in petals and an increased β-cyclase activity,

genetically modified plants which, in comparison with the wild type, have an increased or generated ketolase activity in petals and a reduced ε-cyclase activity,

genetically modified plants which, in comparison with the wild type, have an increased or generated ketolase activity in petals and an increased hydroxylase activity, and an increased β-cyclase activity,

genetically modified plants which, in comparison with the wild type, have an increased or generated ketolase activity in petals and an increased hydroxylase activity, and a reduced ε-cyclase activity,

genetically modified plants which, in comparison with the wild type, have an increased or generated ketolase activity in petals and an increased β-cyclase activity, and a reduced ε-cyclase activity,

genetically modified plants which, in comparison with the wild type, have an increased or generated ketolase activity in petals and an increased hydroxylase activity, and an increased β-cyclase activity and a reduced ε-cyclase activity,

genetically modified plants which, in comparison with the wild type, have an increased or generated ketolase activity in petals, a reduced ε-cyclase activity and an increased β-cyclase activity,

genetically modified plants which, in comparison with the wild type, have an increased or generated ketolase activity in petals, a reduced ε-cyclase activity and a reduced endogenous β-hydroxylase activity,

genetically modified plants which, in comparison with the wild type, have an increased or generated ketolase activity in petals, a reduced ε-cyclase activity and an increased hydroxylase activity,

genetically modified plants which, in comparison with the wild type, have an increased or generated ketolase activity in petals, an increased β-cyclase activity and an increased hydroxylase activity,

genetically modified plants which, in comparison with the wild type, have an increased or generated ketolase activity in petals, an increased β-cyclase activity and a reduced endogenous β-hydroxylase activity,

genetically modified plants which, in comparison with the wild type, have an increased or generated ketolase activity in petals, and an increased β-cyclase activity,

genetically modified plants which, in comparison with the wild type, have an increased or generated ketolase activity in petals, a reduced ε-cyclase activity and at least one further increased activity selected from the group consisting of HMG-CoA reductase activity, (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity, 1-deoxy-D-xylose-5-phosphate synthase activity, 1-deoxy-D-xylose-5-phosphate reductoisomerase activity, isopentenyl-diphosphate Δ-isomerase activity, geranyl-diphosphate synthase activity, farnesyl-diphosphate synthase activity, geranylgeranyl-diphosphate synthase activity, phytoene synthase activity, phytoene desaturase activity, zeta-carotene desaturase activity, crtISO activity, FtsZ activity and MinD activity.

genetically modified plants which, in comparison with the wild type, have an increased or generated ketolase activity in petals, a reduced ε-cyclase activity, an increased β-cyclase activity and an increased hydroxylase activity,

genetically modified plants which, in comparison with the wild type, have an increased or generated ketolase activity in petals, a reduced ε-cyclase activity, an increased β-cyclase activity and a reduced endogenous β-hydroxylase activity,

genetically modified plants which, in comparison with the wild type, have an increased or generated ketolase activity in petals, a reduced ε-cyclase activity and an increased β-cyclase activity,

genetically modified plants which, in comparison with the wild type, have an increased or generated ketolase activity in petals, a reduced ε-cyclase activity and an increased hydroxylase activity,

genetically modified plants which, in comparison with the wild type, have an increased or generated ketolase activity in petals, a reduced ε-cyclase activity and a reduced endogenous β-hydroxylase activity,

genetically modified plants which, in comparison with the wild type, have an increased or generated ketolase activity in petals, a reduced ε-cyclase activity, an increased hydroxylase activity and a reduced endogenous β-hydroxylase activity,

genetically modified plants which, in comparison with the wild type, have an increased or generated ketolase activity in petals, an increased β-cyclase activity, an increased hydroxylase activity and a reduced endogenous β-hydroxylase activity,

genetically modified plants which, in comparison with the wild type, have an increased or generated ketolase activity in petals, a reduced ε-cyclase activity, an increased β-cyclase activity and at least one further increased activity selected from the group consisting of HMG-CoA reductase activity, (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity, 1-deoxy-D-xylose-5-phosphate synthase activity, 1-deoxy-D-xylose-5-phosphate reductoisomerase activity, isopentenyl-diphosphate Δ-isomerase activity, geranyl-diphosphate synthase activity, farnesyl-diphosphate synthase activity, geranylgeranyl-diphosphate synthase activity, phytoene synthase activity, phytoene desaturase activity, zeta-carotene desaturase activity, crtISO activity, FtsZ activity and MinD activity,

genetically modified plants which, in comparison with the wild type, have an increased or generated ketolase activity in petals, a reduced ε-cyclase activity, an increased β-cyclase activity, an increased hydroxylase activity and a reduced endogenous β-hydroxylase activity,

genetically modified plants which, in comparison with the wild type, have an increased or generated ketolase activity in petals, a reduced ε-cyclase activity, an increased β-cyclase activity and an increased hydroxylase activity,

genetically modified plants which, in comparison with the wild type, have an increased or generated ketolase activity in petals, a reduced ε-cyclase activity, an increased β-cyclase activity and a reduced endogenous β-hydroxylase activity,

genetically modified plants which, in comparison with the wild type, have an increased or generated ketolase activity in petals, a reduced ε-cyclase activity, an increased hydroxylase activity and at least one further increased activity, selected from the group consisting of HMG-CoA reductase activity, (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity, 1-deoxy-D-xylose-5-phosphate synthase activity, 1-deoxy-D-xylose-5-phosphate reductoisomerase activity, isopentenyl-diphosphate Δ-isomerase activity, geranyl-diphosphate synthase activity, farnesyl-diphosphate synthase activity, geranylgeranyl-diphosphate synthase activity, phytoene synthase activity, phytoene desaturase activity, zeta-carotene desaturase activity, crtISO activity, FtsZ activity and MinD activity,

genetically modified plants which, in comparison with the wild type, have an increased or generated ketolase activity in petals, a reduced ε-cyclase activity, a reduced endogenous β-hydroxylase activity and at least one further increased activity selected from the group consisting of HMG-CoA reductase activity, (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity, 1-deoxy-D-xylose-5-phosphate synthase activity, 1-deoxy-D-xylose-5-phosphate reductoisomerase activity, isopentenyl-diphosphate Δ-isomerase activity, geranyl-diphosphate synthase activity, farnesyl-diphosphate synthase activity, geranylgeranyl-diphosphate synthase activity, phytoene synthase activity, phytoene desaturase activity, zeta-carotene desaturase activity, crtISO activity, FtsZ activity and MinD activity,

genetically modified plants which, in comparison with the wild type, have an increased or generated ketolase activity in petals, an increased β-cyclase activity, an increased hydroxylase activity and at least one further increased activity selected from the group consisting of HMG-CoA reductase activity, (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity, 1-deoxy-D-xylose-5-phosphate synthase activity, 1-deoxy-D-xylose-5-phosphate reductoisomerase activity, isopentenyl-diphosphate Δ-isomerase activity, geranyl-diphosphate

synthase activity, farnesyl-diphosphate synthase activity, geranylgeranyl-diphosphate synthase activity, phytoene synthase activity, phytoene desaturase activity, zeta-carotene desaturase activity, crtISO activity, FtsZ activity and MinD activity,

genetically modified plants which, in comparison with the wild type, have an increased or generated ketolase activity in petals, an increased β-cyclase activity, a reduced endogenous β-hydroxylase activity and at least one further increased activity selected from the group consisting of HMG-CoA reductase activity, (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity, 1-deoxy-D-xylose-5-phosphate synthase activity, 1-deoxy-D-xylose-5-phosphate reductoisomerase activity, isopentenyl-diphosphate Δ-isomerase activity, geranyl-diphosphate synthase activity, farnesyl-diphosphate synthase activity, geranylgeranyl-diphosphate synthase activity, phytoene synthase activity, phytoene desaturase activity, zeta-carotene desaturase activity, crtISO activity, FtsZ activity and MinD activity,

genetically modified plants which, in comparison with the wild type, have an increased or generated ketolase activity in petals, a reduced ε-cyclase activity, an increased β-cyclase activity and an increased hydroxylase activity and a reduced β-hydroxylase activity,

genetically modified plants which, in comparison with the wild type, have an increased or generated ketolase activity in petals, a reduced ε-cyclase activity, an increased β-cyclase activity, an increased hydroxylase activity and at least one further increased activity selected from the group consisting of HMG-CoA reductase activity, (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity, 1-deoxy-D-xylose-5-phosphate synthase activity, 1-deoxy-D-xylose-5-phosphate reductoisomerase activity, isopentenyl-diphosphate Δ-isomerase activity, geranyl-diphosphate synthase activity, farnesyl-diphosphate synthase activity, geranylgeranyl-diphosphate synthase activity, phytoene synthase activity, phytoene desaturase activity, zeta-carotene desaturase activity, crtISO activity, FtsZ activity and MinD activity,

genetically modified plants which, in comparison with the wild type, have an increased or generated ketolase activity in petals, a reduced ε-cyclase activity, an increased β-cyclase activity, a reduced endogenous β-hydroxylase activity and at least one further increased activity selected from the group consisting of HMG-CoA reductase activity, (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity, 1-deoxy-D-xylose-5-phosphate synthase activity, 1-deoxy-D-xylose-5-phosphate reductoisomerase activity,

isopentenyl-diphosphate Δ-isomerase activity, geranyl-diphosphate synthase activity, farnesyl-diphosphate synthase activity, geranylgeranyl-diphosphate synthase activity, phytoene synthase activity, phytoene desaturase activity, zeta-carotene desaturase activity, crtISO activity, FtsZ activity and MinD activity.

Especially preferred genetically modified plants have, in comparison with the wild type, an increased or generated ketolase activity in petals, an increased β-cyclase activity and an increased hydroxylase activity, where

the increased ketolase activity is caused by introducing nucleic acids which encode a protein comprising the amino acid sequence SEQ ID NO: 2 or a sequence which is derived from this sequence by substitution, insertion or deletion of amino acids and which has at least 20% identity at the amino acid level with the sequence SEQ ID NO: 2 and the enzymatic property of a ketolase,

the increased β-cyclase activity is caused by introducing nucleic acids which encode a β-cyclase comprising the amino acid sequence SEQ ID NO: 96 or a sequence which is derived from this sequence by substitution, insertion or deletion of amino acids and which has at least 20% identity at the amino acid level with the sequence SEQ ID NO: 20,

and the increased hydroxylase activity is caused by introducing nucleic acids which encode a hydroxylase comprising the amino acid sequence SEQ ID NO: 98 or a sequence which is derived from this sequence by substitution, insertion or deletion of amino acids and which has at least 20% identity at the amino acid level with the sequence SEQ ID NO: 18.

Especially preferred genetically modified plants have, in comparison with the wild type, an increased or generated ketolase activity in petals, a reduced ε-cyclase activity, an increased β-cyclase activity, an increased hydroxylase activity and a reduced endogenous β-hydroxylase activity, where

the increased ketolase activity is caused by introducing nucleic acids which encode a protein comprising the amino acid sequence SEQ ID NO: 2 or a sequence which is derived from this sequence by substitution, insertion or deletion of amino acids and which has at least 20% identity at the amino acid level with the sequence SEQ ID NO: 2 and the enzymatic property of a ketolase,

the increased β-cyclase activity is caused by introducing nucleic acids which encode a β-cyclase comprising the amino acid sequence SEQ ID NO: 96 or a sequence which is derived from this sequence by substitution, insertion or deletion of amino acids and which has at least 20% identity at the amino acid level with the sequence SEQ ID NO: 20,

the increased hydroxylase activity is caused by introducing nucleic acids which encode a hydroxylase comprising the amino acid sequence SEQ ID NO: 98 or a sequence which is derived from this sequence by substitution, insertion or deletion of amino acids and which has at least 20% identity at the amino acid level with the sequence SEQ ID NO: 18, and the reduced ε-cyclase activity and a reduced endogenous β-hydroxylase activity in accordance with the above-described, preferred embodiments is produced.

These genetically modified plants can be generated as described hereinbelow, for example by introducing individual nucleic acid constructs (expression cassettes) or by introducing multiple constructs which comprise up to two, three or four of the activities described.

In the method according to the invention for the production of ketocarotenoids, the cultivation step of the genetically modified plants, hereinbelow also referred to as transgenic plants, is preferably followed by harvesting of the plants and isolating ketocarotenoids from the petals of the plants.

The transgenic plants are grown in a manner known per se on substrates and harvested in a suitable manner.

Ketocarotenoids are isolated from the harvested petals in a manner known per se, for example by drying followed by extraction and, if appropriate, further chemical or physical purification processes such as, for example, precipitation methods, crystallography, thermal separation methods such as rectification methods or physical separation methods such as, for example, chromatography. Preferably, for example, ketocarotenoids are isolated from the petals with organic solvents such as acetone, hexane, ether or tert-methyl butyl ether.

Further isolation methods for ketocarotenoids, in particular from petals, are described, for example, in Egger and Kleinig (Phytochemistry (1967) 6, 437-440) and Egger (Phytochemistry (1965) 4, 609-618).

By preference, the ketocarotenoids are selected from the group astaxanthin, canthaxanthin, echinenone, 3-hydroxyechinenone, 3'-hydroxyechinenone, adonirubin and adonixanthin.

An especially preferred ketocarotenoid is astaxanthin.

In the method according to the invention, the ketocarotenoids are generated, in petals, in the form of their mono- or diesters with fatty acids. Examples of some of the fatty acids which have been detected are myristic acid, palmitic acid, stearic acid, oleic acid, linolenic acid and lauric acid (Kamata and Simpson (1987) Comp. Biochem. Physiol. Vol. 86B(3), 587-591).

The production of genetically modified plants with increased or generated ketolase activity in petals is described hereinbelow by way of example. Increasing further activities such as, for example, the hydroxylase activity and/or the β-cyclase activity and/or the HMG-CoA reductase activity and/or the (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity and/or the 1-deoxy-D-xylose-5-phosphate synthase activity and/or the 1-deoxy-D-xylose-5-phosphate reductoisomerase activity and/or the isopentenyl-diphosphate Δ-isomerase activity and/or the geranyl-diphosphate synthase activity and/or the farnesyl-diphosphate synthase activity and/or the geranylgeranyl-diphosphate synthase activity and/or the phytoene synthase activity and/or the phytoene desaturase activity and/or the zeta-carotene desaturase activity and/or the crtISO activity and/or the FtsZ activity and/or the MinD activity can be effected analogously using nucleic acid sequences encoding a hydroxylase or β-cyclase, respectively, or nucleic acids encoding an HMG-CoA reductase and/or nucleic acids encoding an (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase and/or nucleic acids encoding a 1-deoxy-D-xylose-5-phosphate synthase and/or nucleic acids encoding a 1-deoxy-D-xylose-5-phosphate reductoisomerase and/or nucleic acids encoding an isopentenyl-diphosphate Δ-isomerase and/or nucleic acids encoding a geranyl-diphosphate synthase and/or nucleic acids encoding a farnesyl-diphosphate synthase and/or nucleic acids encoding a geranylgeranyl-diphosphate synthase and/or nucleic acids encoding a phytoene synthase and/or nucleic acids encoding a phytoene desaturase and/or nucleic acids encoding a zeta-carotene desaturase and/or nucleic acids encoding a crtISO protein and/or nucleic acids encoding an FtsZ protein and/or nucleic acids encoding a MinD protein, instead of nucleic acid sequences encoding a ketolase. The reduction of further activities such as, for example, the reduction of the ε-cyclase activity, or of the endogenous β-hydroxylase activity, respectively, can be effected analogously using anti-ε-cyclase

nucleic acid sequences or ε-cyclase inverted repeat nucleic acid sequences, or using anti-endogenenous β-hydroxylase nucleic acid sequences or endogenous β-hydroxylase inverted repeat nucleic acid sequences, respectively, instead of nucleic acid sequences encoding a ketolase. In the case of combinations of genetic modifications, the transformation can be carried out individually or using multiple constructs.

The transgenic plants are preferably generated by transforming the starting plants with a nucleic acid construct which comprises the above-described nucleic acids encoding a ketolase which are functionally linked to one or more regulatory signals which ensure the transcription and translation in plants.

These nucleic acid constructs in which the coding nucleic acid sequence is functionally linked to one or more regulatory signals which ensure the transcription and translation in plants are hereinbelow also referred to as expression cassettes.

The invention furthermore relates to nucleic acid constructs comprising at least one nucleic acid encoding a ketolase and additionally at least one further nucleic acid selected from the group consisting of

a) nucleic acids encoding a β-cyclase,
b) nucleic acids encoding a β-hydroxylase,
c) nucleic acids encoding an HMG-CoA reductase,
d) nucleic acids encoding an (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase,
e) nucleic acids encoding a 1-deoxy-D-xylose-5-phosphate synthase,
f) nucleic acids encoding a 1-deoxy-D-xylose-5-phosphate reductoisomerase,
g) nucleic acids encoding an isopentenyl-diphosphate Δ-isomerase,
h) nucleic acids encoding a geranyl-diphosphate synthase,
i) nucleic acids encoding a farnesyl-diphosphate synthase,
j) nucleic acids encoding a geranylgeranyl-diphosphate synthase,
k) nucleic acids encoding a phytoene synthase,
l) nucleic acids encoding a phytoene desaturase,
m) nucleic acids encoding a zeta-carotene desaturase,
n) nucleic acids encoding a crtISO protein,
o) nucleic acids encoding an FtsZ protein,
p) nucleic acids encoding a MinD protein,
q) double-stranded endogenous β-hydroxylase ribonucleic acid sequence and/or endogenous β-hydroxylase antisense ribonucleic acid sequences and

r) double-stranded ε-cyclase ribonucleic acid sequence and/or ε-cyclase antisense ribonucleic acid sequence,

where the nucleic acids are functionally linked to one or more regulatory signals which ensure the transcription and translation in plants.

Increasing or reducing more than four activities using one nucleic acid construct is technically very difficult, in particular in plants. This is why it is preferred to use combinations of nucleic acid constructs in order to increase or reduce the activities, in particular in order to increase or reduce more than 4 activities, in the organism.

However, it is also possible to cross genetically modified organisms which comprise activities which have already been modified. By crossing genetically modified organisms, each of which comprising two modified activities, for example, it is possible to generate organisms with four modified activities. The same can also be achieved by introducing, into the organism, a combination of two nucleic acid constructs, each of which modifies 2 activities.

In a preferred embodiment, the preferred genetically modified organisms are generated by introducing combinations of nucleic acid constructs.

Preferred nucleic acid constructs according to the invention comprise the following combinations of nucleic acids in functional linkage with one or more regulatory signals which ensure the transcription and translation in plants:

ketolase + epsilon
ketolase + beta
ketolase + hydro (OEX)
ketolase + epsilon + beta
ketolase + epsilon + hydro (RNAi)
ketolase + epsilon + hydro (OEX)
ketolase + beta + hydro (RNAi)
ketolase + beta + hydro (OEX)
ketolase + epsilon + (xxx)
ketolase + epsilon + beta + hydro (OEX)
ketolase + epsilon + beta + hydro (RNAi)
ketolase + epsilon + beta
ketolase + epsilon + hydro (OEX)
ketolase + epsilon + hydro (RNAi)
ketolase + epsilon + hydro (OEX) + hydro (RNAi)
ketolase + beta + hydro (OEX) + hydro (RNAi)

ketolase + epsilon + beta + (xxx)
ketolase + epsilon + beta + hydro (OEX) + hydro (RNAi)
ketolase + epsilon + beta + hydro (OEX)
ketolase + epsilon + beta + hydro (RNAi)
ketolase + epsilon + hydro (RNAi) + (xxx)
ketolase + epsilon + hydro (OEX) + (xxx)
ketolase + beta + hydro (RNAi) + (xxx)
ketolase + beta + hydro (OEX) + (xxx)
ketolase + epsilon + beta + hydro (OEX) + hydro (RNAi)
ketolase + epsilon + beta + hydro (OEX) + (xxx)
ketolase + epsilon + beta + hydro (RNAi) + (xxx),

where the abbreviations have the following meanings:

ketolase:	nucleic acids encoding a ketolase
beta:	nucleic acids encoding a β-cyclase
hydro (OEX):	expression of nucleic acids encoding a β-hydroxylase
hydro (RNAi):	double-stranded endogenous β-hydroxylase ribonucleic acid sequence and/or endogenous β-hydroxylase antisense ribonucleic acid sequences
epsilon:	double-stranded ε-cyclase ribonucleic acid sequence and/or ε-cyclase antisense ribonucleic acid sequence
(xxx):	at least one nucleic acid selected from the group consisting of nucleic acids encoding an HMG-CoA reductase, nucleic acids encoding an (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase, nucleic acids encoding a 1-deoxy-D-xylose-5-phosphate synthase, nucleic acids encoding a 1-deoxy-D-xylose-5-phosphate reductoisomerase, nucleic acids encoding an isopentenyl-diphosphate Δ-isomerase, nucleic acids encoding a geranyl-diphosphate synthase, nucleic acids encoding a farnesyl-diphosphate synthase, nucleic acids encoding a geranylgeranyl-diphosphate synthase, nucleic acids encoding a phytoene synthase, nucleic acids encoding a phytoene desaturase, nucleic acids encoding a zeta-carotene desaturase, nucleic acids encoding a crtISO protein, nucleic acids encoding an FtsZ protein and nucleic acids encoding a MinD protein.

By preference, the regulatory signals comprise one or more promoters which ensure the transcription and translation in plants.

The expression cassettes comprise regulatory signals, i.e. regulatory nucleic acid sequences which regulate the expression of the coding sequence in the host cell. In accordance with a preferred embodiment, an expression cassette comprises upstream, i.e. at the 5'terminus of the coding sequence, a promoter and downstream, i.e. at the 3'terminus, a polyadenylation signal and, if appropriate, further regulatory elements which are linked operably with the interjacent coding sequence for at least one of the above-described genes. Operable linkage is understood as meaning the sequential arrangement of promoter, coding sequence, terminator and, if appropriate, further regulatory elements in such a way that each of the regulatory elements can fulfil its intended function when the coding sequence is expressed.

The preferred nucleic acid constructs, expression cassettes and vectors for plants and methods for producing transgenic plants, and the transgenic plants themselves, are described hereinbelow by way of example.

The sequences which are preferred for the operable linkage, but not limited thereto, are targeting sequences for ensuring the subcellular localization in the apoplast, in the vacuole, in plastids, in the mitochondrium, in the endoplasmic reticulum (ER), in the nucleus, in oil bodies or other compartments, and translation enhancers such as the tobacco mosaic virus 5'-leader sequence (Gallie et al., Nucl. Acids Res. 15 (1987), 8693-8711).

In principle, any promoter which is capable of controlling the expression of foreign genes in plants is suitable as promoter of the expression cassette.

"Constitutive" promoter means those promoters which ensure expression in a large number of, preferably all, tissues over a substantial period of the plant's development, preferably at all points in time of the plant's development.

A promoter which is used by preference is, in particular, a plant promoter or a promoter derived from a plant virus. Especially preferred is the promoter of the CaMV cauliflower mosaic virus 35S transcript (Franck et al. (1980) Cell 21:285-294; Odell et al. (1985) Nature 313:810-812; Shewmaker et al. (1985) Virology 140:281-288; Gardner et al. (1986) Plant Mol Biol 6:221-228) or the 19S CaMV promoter (US 5,352,605; WO 84/02913; Benfey et al. (1989) EMBO J 8:2195-2202).

A further suitable constitutive promoter is the pds promoter (Pecker et al. (1992) Proc. Natl. Acad. Sci USA 89: 4962-4966) or the "Rubisco small subunit (SSU)" promoter (US 4,962,028), the legumin B promoter (GenBank Acc. No. X03677), the promoter of the Agrobacterium nopaline synthase, the TR dual promoter, the OCS (octopine synthase) promoter from Agrobacterium, the ubiquitin promoter (Holtorf S et al. (1995) Plant Mol Biol 29:637-649), the ubiquitin 1 promoter (Christensen et al. (1992) Plant Mol Biol 18:675-689; Bruce et al. (1989) Proc Natl Acad Sci USA 86:9692-9696), the Smas promoter, the cinnamyl alcohol dehydrogenase promoter (US 5,683,439), the promoters of the vacuolar ATPase subunits or the promoter of a proline-rich protein from wheat (WO 91/13991), the Pnit promoter (Y07648.L, Hillebrand et al. (1998), Plant. Mol. Biol. 36, 89-99, Hillebrand et al. (1996), Gene, 170, 197-200, the ferredoxin NADPH oxidoreductase promoter (database entry AB011474, position 70127 to 69493), the TPT promoter (WO 03006660), the "superpromoter" (US Patent 5955646), the 34S promoter (US Patent 6051753), and further promoters of genes whose constitutive expression in plants is known to the skilled worker.

The expression cassettes may also comprise a chemically inducible promoter (review paper: Gatz et al. (1997) Annu Rev Plant Physiol Plant Mol Biol 48:89-108), by means of which the expression of the ketolase gene in the plant can be controlled at a particular point in time. Such promoters such as, for example, the PRP1 promoter (Ward et al. (1993) Plant Mol Biol 22:361-366), salicylic-acid-inducible promoter (WO 95/19443), a benzene-sulfonamide-inducible promoter (EP 0 388 186), a tetracyclin-inducible promoter (Gatz et al. (1992) Plant J 2:397-404), an abscisic-acid-inducible promoter (EP 0 335 528) or an ethanol- or cyclohexanone-inducible promoter (WO 93/21334) can likewise be used.

Other preferred promoters are those which are induced by biotic or abiotic stress such as, for example, the pathogen-inducible promoter of the PRP1 gene (Ward et al. (1993) Plant Mol Biol 22:361-366), the heat-inducible hsp70 or hsp80 promoter from tomato (US 5,187,267), the cold-inducible alpha-amylase promoter from potato (WO 96/12814), the light-inducible PPDK promoter or the wounding-induced pinII promoter (EP375091).

Pathogen-inducible promoters comprise the promoters of genes which are induced as the result of a pathogen attack such as, for example, genes of PR proteins, SAR proteins, β-1,3-glucanase, chitinase and the like (for example Redolfi et al. (1983) Neth J Plant Pathol 89:245-254; Uknes, et al. (1992) The Plant Cell

4:645-656; Van Loon (1985) Plant Mol Viral 4:111-116; Marineau et al. (1987) Plant Mol Biol 9:335-342; Matton et al. (1987) Molecular Plant-Microbe Interactions 2:325-342; Somssich et al. (1986) Proc Natl Acad Sci USA 83:2427-2430; Somssich et al. (1988) Mol Gen Genetics 2:93-98; Chen et al. (1996) Plant J 10:955-966; Zhang and Sing (1994) Proc Natl Acad Sci USA 91:2507-2511; Warner, et al. (1993) Plant J 3:191-201; Siebertz et al. (1989) Plant Cell 1:961-968(1989).

Also comprised are wounding-inducible promoters such as that of the promoter of the pinII gene (Ryan (1990) Ann Rev Phytopath 28:425-449; Duan et al. (1996) Nat Biotech 14:494-498), of the wun1 and wun2 gene (US 5,428,148), of the win1 and win2 gene (Stanford et al. (1989) Mol Gen Genet 215:200-208), of the systemin (McGurl et al. (1992) Science 225:1570-1573), of the WIP1 gene (Rohmeier et al. (1993) Plant Mol Biol 22:783-792; Ekelkamp et al. (1993) FEBS Letters 323:73-76), of the MPI gene (Corderok et al. (1994) The Plant J 6(2):141-150) and the like.

Further suitable promoters are, for example, fruit-maturation-specific promoters such as, for example, the fruit-maturation-specific promoter from tomato (WO 94/21794, EP 409 625). Some of the promoters which the development-promoters comprise are the tissue-specific promoters since, naturally, the individual tissues are formed as a function of the development.

Furthermore preferred are in particular those promoters which ensure the expression in tissues or plant parts in which, for example, the biosynthesis of ketocarotenoids or their precursors takes place. Examples of preferred promoters are promoters with specificities for the anthers, ovaries, petals, sepals, flowers, leaves, stems and roots and combinations hereof.

Tuber-specific, storage-root-specific or root-specific promoters are, for example, the patatin promoter class I (B33) or the promoter of the cathepsin D inhibitor from potato.

Examples of leaf-specific promoters are, for example, the promoter of the cytosolic FBPase from potato (WO 97/05900), the SSU promoter (small subunit) of Rubisco (ribulose-1,5-bisphosphate carboxylase) or the ST-LSI promoter from potato (Stockhaus et al. (1989) EMBO J 8:2445-2451).

Examples of flower-specific promoters are the phytoene synthase promoter (WO 92/16635), the promoter of the P-rr gene (WO 98/22593), the EPSPS promoter (database entry M37029), the DFR-A promoter (database entry X79723), the B gene promoter

(WO 0008920) and the CHRC promoter (WO 98/24300; Vishnevetsky et al. (1996) Plant J. 10, 1111-1118), and the promoters of the Arabidopsis gene loci At5g33370 (hereinbelow M1 promoter), At5g22430 (hereinbelow M2 promoter) and At1g26630 (hereinbelow M3 promoter).

Examples of anther-specific promoters are the 5126 promoter (US 5,689,049, US 5,689,051), the glob-1 promoter or the g-zein promoter.

Further promoters which are suitable for expression in plants are described in Rogers et al. (1987) Methods in Enzymol 153:253-277; Schardl et al. (1987) Gene 61:1-11 and Berger et al. (1989) Proc Natl Acad Sci USA 86:8402-8406.

As a rule, all of the promoters described in the present application make possible the expression of ketolase in petals of the plants according to the invention.

Especially preferred in the method according to the invention are constitutive flower-specific and, in particular, petal-specific promoters.

The present invention therefore relates in particular to a nucleic acid construct comprising, in functional linkage, a flower-specific or, in particular, a petal-specific promoter and a nucleic acid encoding a ketolase.

An expression cassette is prefereably prepared by fusing a suitable promoter with an above-described nucleic acid encoding a ketolase and preferably a nucleic acid which is inserted between promoter and nucleic acid sequence and which encodes a plastid-specific transit peptide, and with a polyadenylation signal, using customary recombination and cloning techniques as are described, for example, in T. Maniatis, E.F. Fritsch and J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989) and in T.J. Silhavy, M.L. Berman und L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and in Ausubel, F.M. et al., Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley-Interscience (1987).

The nucleic acids which encode a plastidic transit peptide and which are preferably inserted ensure the localization in plastids and in particular in chromoplasts.

It is also possible to use expression cassettes whose nucleic acid sequence encodes a ketolase fusion protein, where part of the fusion protein is a transit peptide which governs the translocation of the polypeptide. Preferred are chromoplast-specific transit peptides which are cleaved enzymatically from the ketolase moiety after translocation of the ketolase into the chromoplasts.

Especially preferred is the transit peptide which is derived from the plastidic *Nicotiana tabacum* transketolase or from another transit peptide (for example the transit peptide of the Rubisco small subunit (rbcS) or the transit peptide of the ferredoxin-NADP oxidoreductase and of the isopentenyl-pyrophosphate isomerase-2) or its functional equivalent.

Particularly preferred are nucleic acid sequences of three cassettes of the plastid transit peptide of the tobacco plastidic transketolase in three reading frames as KpnI/BamHI fragments with an ATG codon in the NcoI cleavage site:

pTP09

KpnI_GGTACCATGGCGTCTTCTTCTTCTCTCACTCTCTCTCAAGCTATCCTCTCTCGTTCTGTC
CCTCGCCATGGCTCTGCCTCTTCTTCTCAACTTTCCCCTTCTTCTCTCACTTTTTCCGGCCTTAA
ATCCAATCCCAATATCACCACCTCCCGCCGCCGTACTCCTTCCTCCGCCGCCGCCGCCGCCGTCG
TAAGGTCACCGGCGATTCGTGCCTCAGCTGCAACCGAAACCATAGAGAAAACTGAGACTGCGGGA
TCC_BamHI

pTP10

KpnI_GGTACCATGGCGTCTTCTTCTTCTCTCACTCTCTCTCAAGCTATCCTCTCTCGTTCTGTC
CCTCGCCATGGCTCTGCCTCTTCTTCTCAACTTTCCCCTTCTTCTCTCACTTTTTCCGGCCTTAA
ATCCAATCCCAATATCACCACCTCCCGCCGCCGTACTCCTTCCTCCGCCGCCGCCGCCGCCGTCG
TAAGGTCACCGGCGATTCGTGCCTCAGCTGCAACCGAAACCATAGAGAAAACTGAGACTGCGCTG
GATCC_BamHI

pTP11

KpnI_GGTACCATGGCGTCTTCTTCTTCTCTCACTCTCTCTCAAGCTATCCTCTCTCGTTCTGTC
CCTCGCCATGGCTCTGCCTCTTCTTCTCAACTTTCCCCTTCTTCTCTCACTTTTTCCGGCCTTAA
ATCCAATCCCAATATCACCACCTCCCGCCGCCGTACTCCTTCCTCCGCCGCCGCCGCCGCCGTCG
TAAGGTCACCGGCGATTCGTGCCTCAGCTGCAACCGAAACCATAGAGAAAACTGAGACTGCGGGG
ATCC_BamHI

Further examples of a plastidic transit peptide are the transit peptide of the plastidic isopentenyl-pyrophosphate isomerase-2 (IPP-2) from Arabidopsis thaliana and the transit peptide of the

ribulose-bisphosphate carboxylase small subunit (rbcS) from pea (Guerineau, F, Woolston, S, Brooks, L, Mullineaux, P (1988) An expression cassette for targeting foreign proteins into the chloroplasts. Nucl. Acids Res. 16: 11380).

The nucleic acids according to the invention can be generated synthetically or obtained naturally or comprise a mixture of synthetic and natural nucleic acid constituents, and consist of various heterologous gene segments from a variety of organisms.

Preferrred are, as described above, synthetic nucleotide sequences with codons which are preferred by plants. These codons which are preferred by plants can be determined from codons with the highest protein frequency which are expressed in most of the plant species of interest.

When preparing an expression cassette, various DNA fragments can be manipulated in order to obtain a nucleotide sequence which expediently reads in the correct direction and is equipped with a correct reading frame. To link the DNA fragments to one another, adaptors or linkers may be added to the fragments.

Expediently, the promoter and the terminator regions can be provided, in the direction of transcription, with a linker or polylinker comprising one or more restriction sites for the insertion of this sequence. As a rule, the linker has 1 to 10, in most cases 1 to 8, preferably 2 to 6, restriction sites. In general, the linker has a size of less than 100 bp, frequently less than 60 bp, but at least 5 bp, within the regulatory regions. The promoter can either be native, or homologous, or else foreign, or heterologous, to the host plant. Preferably, the expression cassette comprises, in the 5'-3' direction of transcription, the promoter, a coding nucleic acid sequence or a nucleic acid construct and a region for transcriptional termination. Various termination regions can be exchanged for one another as desired.

Examples of a terminator are the 35S terminator (Guerineau et al. (1988) Nucl Acids Res. 16: 11380), the nos terminator (Depicker A, Stachel S, Dhaese P, Zambryski P, Goodman HM. Nopaline synthase: transcript mapping and DNA sequence. J Mol Appl Genet. 1982;1(6):561-73) or the ocs terminator (Gielen, J, de Beuckeleer, M, Seurinck, J, Debroek, H, de Greve, H, Lemmers, M, van Montagu, M, Schell, J (1984) The complete sequence of the TL-DNA of the Agrobacterium tumefaciens plasmid pTiAch5. EMBO J. 3: 835-846).

Furthermore, it is possible to employ manipulations which provide suitable restriction cleavage sites or which remove superfluous DNA or restriction cleavage sites. Where insertions, deletions or substitutions such as, for example, transitions and transversions are suitable, it is possible to use *in-vitro* mutagenesis, primer repair, restriction or ligation.

In the case of suitable manipulations such as, for example, restriction, chewing-back or filling up overhangs for blunt ends, it is possible to provide complementary ends of the fragments for the ligation.

Preferred polyadenylation signals are plant polyadenylation signals, preferably those which correspond essentially to T-DNA polyadenylation signals from Agrobacterium tumefaciens, in particular the gene 3 of the T-DNA (octopine synthase) of the Ti plasmid pTiACH5 (Gielen et al., EMBO J. 3 (1984), 835 et seq.), or functional equivalents.

The transfer of foreign genes in the genome of a plant is referred to as transformation.

To this end, it is possible to exploit methods which are known per se for the transformation and regeneration of plants from plant tissues or plant cells in order to carry out a transient or stable transformation.

Suitable methods for the transformation of plants are the transformation of protoplasts by means of polyethylene-glycol-induced DNA uptake, the biolistic method using the gene gun - what is known as the particle bombardment method, electroporation, incubation of dry embryos in DNA-comprising solution, microinjection, and the above-described Agrobacterium-mediated gene transfer. The above methods are described, for example, in B. Jenes et al., Techniques for Gene Transfer, in: Transgenic Plants, Vol. 1, Engineering and Utilization, edited by S.D. Kung and R. Wu, Academic Press (1993), 128-143 and in Potrykus, Annu. Rev. Plant Physiol. Plant Molec. Biol. 42 (1991), 205-225).

By preference, the construct to be expressed is cloned into a vector which is suitable for the transformation of Agrobacterium tumefaciens, for example pBin19 (Bevan et al., Nucl. Acids Res. 12 (1984), 8711) or particularly preferably pSUN2, pSUN3, pSUN4 or pSUN5 (WO 02/00900).

Agrobacteria which have been transformed with an expression plasmid can be used in the known manner for the transformation of plants, for example by bathing scarified leaves or leaf segments in an agrobacterial solution and subsequently growing them in suitable media.

For the preferred generation of genetically modified plants, hereinbelow also referred to as transgenic plants, the fused expression cassette which expresses a ketolase is cloned into a vector, for example pBin19 or, in particular, pSUN2, which is suitable for being transformed into *Agrobacterium tumefaciens*. Agrobacteria which have been transformed with such a vector can then be used in the known manner for the transformation of plants, in particular crop plants, for example by bathing scarified leaves or leaf segments in an agrobacterial solution and subsequently growing them in suitable media.

The transformation of plants by agrobacteria is known, inter alia, from F.F. White, Vectors for Gene Transfer in Higher Plants; in Transgenic Plants, Vol. 1, Engineering and Utilization, edited by S.D. Kung and R. Wu, Academic Press, 1993, pp. 15-38. Transgenic plants can be regenerated in the known manner from the transformed cells of the scarified leaves or leaf segments, and such plants comprise a gene for the expression of a nucleic acid encoding a ketolase integrated into the expression cassette.

To transform a host plant with a nucleic acid which encodes a ketolase, an expression cassette is incorporated, as insertion, into a recombinant vector whose vector DNA comprises additional functional regulatory signals, for example sequences for replication or integration. Suitable vectors are described, inter alia, in "Methods in Plant Molecular Biology and Biotechnology" (CRC Press), chapter 6/7, pp. 71-119 (1993).

Using the above-cited recombination and cloning techniques, the expression cassettes can be cloned into suitable vectors which make possible their multiplication, for example in *E. coli*. Suitable cloning vectors are, inter alia, pJIT117 (Guerineau et al. (1988) Nucl. Acids Res.16 :11380), pBR332, pUC series, M13mp series and pACYC184. Especially suitable are binary vectors, which are capable of replication both in *E. coli* and in agrobacteria.

In this context, expression can take place constitutively or, preferably, specifically in the petals, depending on the choice of the promoter.

Accordingly, the invention furthermore relates to a method for the production of genetically modified plants, wherein a nucleic acid construct comprising, in functional linkage, a flower-specific promoter and nucleic acids encoding a ketolase is introduced into the genome of the starting plant.

The invention furthermore relates to the genetically modified plants, where the genetic modification

A in the event that the wild-type plant already shows ketolase activity in the petals, increases the activity of a ketolase in petals in comparison with the wild type, and

B in the event that the wild-type plant shows no ketolase activity in petals, produces the activity of a ketolase in petals in comparison with the wild type.

As detailed hereinabove, increasing or producting the ketolase activity in comparison with the wild type is preferably effected by increasing or producting the gene expression of a nucleic acid encoding a ketolase.

In a further preferred embodiment, increasing or producing the gene expression of a nucleic acid encoding a ketolase is effected, as described hereinabove, by introducing, into the plants, nucleic acids encoding a ketolase and thus preferably by overexpressing or transgenically expressing nucleic acids encoding a ketolase.

Preferred transgenic plants which as the wild type show no ketolase activity in the petals comprise, as mentioned hereinabove, at least one transgenic nucleic acid encoding a ketolase.

Especially preferred genetically modified plants additionally show, as mentioned hereinabove, an increased hydroxylase activity and/or β-cyclase activity in comparison with a wild-type plant. Further preferred embodiments are described hereinabove in the method according to the invention.

Further preferred genetically modified plants additionally show, as mentioned hereinabove, a reduced ε-cyclase activity in comparison with a wild-type plant. Further preferred embodiments

are described hereinabove in the method according to the invention.

Further especially preferred genetically modified plants additionally show, as mentioned hereinabove, at least one further increased activity selected from the group consisting of HMG-CoA reductase activity, (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate reductase activity, 1-deoxy-D-xylose-5-phosphate synthase activity, 1-deoxy-D-xylose-5-phosphate reductoisomerase activity, isopentenyl-diphosphate Δ-isomerase activity, geranyl-diphosphate synthase activity, farnesyl-diphosphate synthase activity, geranylgeranyl-diphosphate synthase activity, phytoene synthase activity, phytoene desaturase activity, zeta-carotene desaturase activity, crtISO activity, FtsZ activity and MinD activity, in comparison with the wild-type. Further preferred embodiments are described hereinabove in the method according to the invention.

Further especially preferred genetically modified plants additionally show, as mentioned hereinabove, a reduced endogenous β-hydroxylase activity in comparison with the wild-type. Further preferred embodiments are described hereinabove in the method according to the invention.

In accordance with the invention, plants are preferably understood as meaning plants which, as the wild type, have chromoplasts in petals. Further preferred plants have, as the wild type, additionally carotenoids, in particular β-carotene, zeaxanthin, violaxanthin or lutein in the petals. Further preferred plants additionally have, as the wild type, a β-cyclase activity in the petals. Further preferred plants additionally have, as the wild type, a hydroxylase activity in the petals.

Especially preferred plants are plants selected from the families Ranunculaceae, Berberidaceae, Papaveraceae, Cannabaceae, Rosaceae, Fabaceae, Linaceae, Vitaceae, Brassicaceae, Cucurbitaceae, Primulaceae, Caryophyllaceae, Amaranthaceae, Gentianaceae, Geraniaceae, Caprifoliaceae, Oleaceae, Tropaeolaceae, Solanaceae, Scrophulariaceae, Asteraceae, Liliaceae, Amaryllidaceae, Poaceae, Orchidaceae, Malvaceae, Illiaceae or Lamiaceae.

The invention therefore relates in particular to genetically modified plants selected from the families Ranunculaceae, Berberidaceae, Papaveraceae, Cannabaceae, Rosaceae, Fabaceae, Linaceae, Vitaceae, Brassiceae, Cucurbitaceae, Primulaceae, Caryophyllaceae, Amaranthaceae, Gentianaceae, Geraniaceae, Caprifoliaceae, Oleaceae, Tropaeolaceae, Solanaceae,

Scrophulariaceae, Asteraceae, Liliaceae, Amaryllidaceae, Poaceae, Orchidaceae, Malvaceae, Illiaceaae or Lamiaceae comprising at least one transgenic nucleic acid encoding a ketolase.

Very especially preferred genetically modified plants are selected from the plant genera Marigold, Tagetes erecta, Tagetes patula, Adonis, Lycopersicon, Rosa, Calendula, Physalis, Medicago, Helianthus, Chrysanthemum, Aster, Tulipa, Narcissus, Petunia, Geranium or Tropaeolum, where the genetically modified plant comprises at least one transgenic nucleic acid encoding a ketolase.

In preferred transgenic plants - as mentioned above - the ketolase is expressed in petals; especially preferably, the expression of the ketolase is highest in petals.

The present invention furthermore relates to the transgenic plants, their propagation material and their plant cells, tissues or parts, in particular their petals.

As described above, the genetically modified plants can be used for the production of ketocarotenoids, in particular astaxanthin.

Genetically modified plants according to the invention which can be consumed by humans and animals and which have an increased ketocarotenoid content can also be used for example directly or after processing known per se as foodstuff or feedstuff, or else as food or feed supplement. Furthermore, the genetically modified plants can be used for the production of ketocarotenoid-comprising extracts of the plants and/or for the production of feed and food supplements.

The genetically modified plants can also be used in the field of horticulture as ornamentals.

The genetically modified plants have an increased ketocarotenoid content in comparison with the wild type.

An increased ketocarotenoid content is, as a rule, understood as meaning an increased total ketocarotenoid content.

However, an increased ketocarotenoid content is also understood as meaning, in particular, a modified content of the preferred ketocarotenoids without the total carotenoid content necessarily having to be increased.

In an especially preferred embodiment, the genetically modified plants according to the invention have an increased astaxanthin content in comparison with the wild type.

In this case, an increased content is also understood as meaning a generated content of ketocarotenoids or astaxanthin.

The invention is now illustrated by the examples which follow, but not limited thereto:

General experimental conditions:
Sequence analysis of recombinant DNA

Recombinant DNA molecules were sequenced using a laser fluorescence DNA sequencer from Licor (available from MWG Biotech, Ebersbach) following the method of Sanger (Sanger et al., Proc. Natl. Acad. Sci. USA 74 (1977), 5463-5467).

Example 1:
Amplification of a cDNA which encodes the entire primary sequence of the ketolase from *Haematococcus pluvialis* Flotow em. Wille

The cDNA which encodes the ketolase from *Haematococcus pluvialis* was amplified from *Haematococcus pluvialis* (strain 192.80 of the "Sammlung von Algenkulturen der Universität Göttingen" [Collection of algal cultures of the university of Göttingen]) suspension culture by means of PCR.

To prepare total RNA from a suspension culture of *Haematococcus pluvialis* (strain 192.80) which had grown for 2 weeks with indirect daylight at room temperature in *Haematococcus* medium (1.2 g/l sodium acetate, 2 g/l yeast extract, 0.2 g/l $MgCl_2x6H_2O$, 0.02 $CaCl_2x2H_2O$; pH 6.8; after autoclaving addition of 400 mg/l L-asparagine, 10 mg/l $FeSO_4xH_2O$), the cells were harvested, frozen in liquid nitrogen and ground to a powder in a mortar. Thereafter, 100 mg of the frozen pulverized algal cells were transferred into a reaction vessel and taken up in 0.8 ml of Trizol buffer (LifeTechnologies). The suspension was extracted with 0.2 ml of chloroform. After centrifugation for 15 minutes at 12 000 g, the aqueous supernatant was removed, transferred into a fresh reaction vessel and extracted with one volume of ethanol. The RNA was precipitated with one volume of isopropanol, washed with 75% of ethanol, and the pellet was dissolved in DEPC water (overnight-incubation of water with 1/1000 volume diethyl pyrocarbonate at room temperature, then autoclaving). The RNA concentration was determined photometrically.

For the cDNA synthesis, 2.5 ug of total RNA were denatured for 10 min at 60°C, cooled on ice for 2 minutes and transcribed into cDNA by means of a cDNA kit (Ready-to-go-you-prime-beads, Pharmacia Biotech) following the manufacturer's instructions and using an antisense-specific primer (PR1 SEQ ID NO: 29).

The nucleic acid encoding a ketolase from *Haematococcus pluvialis* (strain 192.80) was amplified by means of polymerase chain reaction (PCR) from *Haematococcus pluvialis* using a sense-specific primer (PR2 SEQ ID NO: 30) and an antisense-specific primer (PR1 SEQ ID NO: 29).

The PCR conditions were as follows:

The PCR for the amplification of the cDNA which encodes a ketolase protein consisting of the entire primary sequence was carried out in 50 µl of reaction mixture comprising:

- 4 µl of a *Haematococcus pluvialis* cDNA (prepared as described above)
- 0.25 mM dNTPs
- 0.2 mM PR1 (SEQ ID NO: 29)
- 0.2 mM PR2 (SEQ ID NO: 30)
- 5 µl 10X PCR buffer (TAKARA)
- 0.25 µl R Taq polymerase (TAKARA)
- 25.8 µl distilled water.

The PCR was carried out under the following cycling conditions:

1X	94°C	2 minutes
35X	94°C	1 minute
	53°C	2 minutes
	72°C	3 minutes
1X	72°C	10 minutes

The PCR amplification with SEQ ID NO: 29 and SEQ ID NO: 30 results in a 1155 bp fragment which encodes a protein consisting of the entire primary sequence (SEQ ID NO: 22). Using standard methods, the amplificate was cloned into the PCR cloning vector pGEM-Teasy (Promega), giving rise to the clone pGKETO2.

Sequencing the clone pGKETO2 with the T7 and the SP6 primer confirmed a sequence which differs from the published sequence X86782 only in the three codons 73, 114 and 119 in, in each case, one base. These nucleotide substitutions were reproduced in an independent amplification experiment and thus represent the

nucleotide sequence in the used *Haematococcus pluvialis* strain 192.80 (Figures 3 and 4, sequence alignments).

This clone was therefore used for cloning into the expression vector pJIT117 (Guerineau et al. 1988, Nucl. Acids Res. 16: 11380). Cloning was effected by isolating the 1027 bp SpHI fragment from pGEM-Teasy and ligation into the SpHI-cut vector pJIT117. The clone which comprises the *Haematococcus pluvialis* ketolase in the correct orientation as N-terminal translational fusion with the rbcs transit peptide is named pJKETO2.

Example 2:
Amplification of a cDNA which encodes the ketolase from *Haematococcus pluvialis* Flotow em. Wille which is truncated at the N-terminus by 14 amino acids

The cDNA which encodes the ketolase from *Haematococcus pluvialis* (strain 192.80) which is truncated at the N terminus by 14 amino acids was amplified by means of PCR from *Haematococcus pluvialis* suspension culture (strain 192.80 of the "Sammlung von Algenkulturen der Universität Göttingen").

The preparation of total RNA from a suspension culture of *Haematococcus pluvialis* (strain 192.80) was carried out as described in Example 1.

The cDNA synthesis was carried out as described in Example 1.

The nucleic acid encoding a ketolase from *Haematococcus pluvialis* (strain 192.80) which is truncated at the N-terminus by 14 amino acids was amplified by means of polymerase chain reaction (PCR) from *Haematococcus pluvialis* using a sense-specific primer (PR3 SEQ ID NO: 31) and an antisense-specific primer (PR1 SEQ ID NO: 29).

The PCR conditions were as follows:

The PCR for the amplification of the cDNA which encodes a ketolase protein which is truncated at the N-terminus by 14 amino acids was carried out in 50 µl of reaction mixture comprising:

- 4 µl of a *Haematococcus pluvialis* cDNA (prepared as described above)
- 0.25 mM dNTPs
- 0.2 mM PR1 (SEQ ID NO: 29)
- 0.2 mM PR2 (SEQ ID NO: 31)
- 5 µl 10X PCR buffer (TAKARA)

- 0.25 µl R Taq polymerase (TAKARA)
- 25.8 µl distilled water.

The PCR was carried out under the following cycling conditions:

1X	94°C	2 minutes
35X	94°C	1 minute
	53°C	2 minutes
	72°C	3 minutes
1X	72°C	10 minutes

The PCR amplification with SEQ ID NO: 29 and SEQ ID NO: 31 resulted in a 1111 bp fragment which encodes a ketolase protein in which N-terminal amino acids (positions 2-16) are replaced by a single amino acid (leucin).

The amplificate was cloned into the PCR cloning vector pGEM-Teasy (Promega) using standard methods. Sequencing reactions with the primers T7 and SP6 confirmed a sequence which is identical to the sequence SEQ ID NO: 22, the 5′ region (positions 1-53) of SEQ ID NO: 22 in the amplificate SEQ ID NO: 24 having been replaced by a nonamer sequence whose sequence deviates. This clone was therefore used for cloning into the expression vector pJIT117 (Guerineau et al. 1988, Nucl. Acids Res. 16: 11380).

Cloning was carried out by isolating the 985 bp SpHI fragment from pGEM-Teasy and ligation with the SpHI-cut vector pJIT117. The clone which comprises the *Haematococcus pluvialis* ketolase which is truncated at the N terminus by 14 amino acids in the correct orientation as N-terminal translational fusion with the rbcs transit peptide is named pJKETO3.

Example 3:
Amplification of a cDNA which encodes the ketolase from *Haematococcus pluvialis* Flotow em. Wille (strain 192.80 of "Sammlung von Algenkulturen der Universität Göttingen") consisting of the entire primary sequence and fused C-terminal myc tag.

The cDNA which encodes the ketolase from *Haematococcus pluvialis* (strain 192.80) consisting of the entire primary sequence and fused C-terminal myc tag was prepared by means of PCR using the plasmid pGKETO2 (described in Example 1) and the primers PR15 (SEQ ID NO: 32). The primer PR15 is compsed of an antisense-specific 3′ region (nucleotides 40 to 59) and a myc-tag encoding 5′ region (nucleotides 1 to 39).

Denaturing (5 min at 95°C) and annealing (slow cooling at room temperature to 40°C) of pGKETO2 and PR15 took place in an 11.5 µl reaction mixture comprising:

- 1 µg pGKETO2 PlasmidDNA
- 0.1 µg PR15 (SEQ ID NO: 32)

The 3' ends were filled in (30 min at 30°C) in 20 µl of reaction mixture comprising:

- 11.5 µl pGKETO2/PR15 annealing reaction (prepared as described above)
- 50 µM dNTPs
- 2 µl 1X Klenow buffer
- 2U Klenow enzyme

The nucleic acid encoding a ketolase from *Haematococcus pluvialis* (strain 192.80) consisting of the entire primary sequence and fused C-terminal myc tag was amplified from *Haematococcus pluvialis* by means of polymerase chain reaction (PCR) using a sense-specific primer (PR2 SEQ ID NO: 30) and an antisense-specific primer (PR15 SEQ ID NO: 32).

The PCR conditions were as follows:

The PCR for the amplification of the cDNA which encodes a ketolase protein with fused C-terminal myc tag was carried out in 50 µl of reaction mixture comprising:

- 1 µl of an annealing reaction (prepared as described above)
- 0.25 mM dNTPs
- 0.2 mM PR15 (SEQ ID NO: 32)
- 0.2 mM PR2 (SEQ ID NO: 30)
- 5 µl 10X PCR buffer (TAKARA)
- 0.25 µl R Taq polymerase (TAKARA)
- 25.8 µl distilled water.

The PCR was carried out under the following cycling conditions:

1X	94°C	2 minutes
35X	94°C	1 minute
	53°C	1 minute
	72°C	1 minute
1X	72°C	10 minutes

The PCR amplification with SEQ ID NO: 32 and SEQ ID NO: 30 results in a 1032 bp fragment which encodes a protein consisting of the entire primary sequence of the ketolase from *Haematococcus pluvialis* as double translational fusion with the rbcS transit peptide at the N terminus und the myc tag at the C terminus.

The amplificate was cloned into the PCR cloning vector pGEM-Teasy (Promega) using standard methods. Sequencing reactions with the primers T7 and SP6 confirmed a sequence which was identical to the sequence SEQ ID NO: 22, the 3' region (positions 993 to 1155) of SEQ ID NO: 22 in the amplificate SEQ ID NO: 26 having been replaced by a 39 bp sequence which deviated. This clone was therefore used for cloning into the expression vector pJIT117 (Guerineau et al. 1988, Nucl. Acids Res. 16: 11380).

Cloning was effected by isolating the 1038 bp EcoRI/SpHI fragment from pGEM-Teasy and ligation with the EcoRI-SpHI-cut vector pJIT117. The ligation gives rise to a translational fusion between the C terminus of the rbcS transit peptide sequence and the N terminus of the ketolase sequence. The clone which comprises the *Haematococcus pluvialis* ketolase with fused C-terminal myc tag in correct orientation as translational N-terminal fusion with the rbcs transit peptide is named pJKETO4.

Example 4:
Preparation of expression vectors for the constitutive expression of the *Haematococcus pluvialis* ketolase in *Lycopersicon esculentum* and *Tagetes erecta*.

Expression of the ketolase from *Haematococcus pluvialis* in *L. esculentum* and in *Tagetes erecta* was under the control of the constitutive promoter d35S from CaMV (Franck et al. 1980, Cell 21: 285-294). The expression was carried out with the transit peptide rbcS from pea (Anderson et al. 1986, Biochem J. 240:709-715).

An expression cassette for the agrobacterium-mediated transformation of the ketolase from *Haematococcus pluvialis* in *L. esculentum* was prepared using the binary vector pSUN3 (WO02/00900).

- To prepare the expression vector pS3KETO2, the 2.8 kb SacI/XhoI fragment from pJKETO2 was ligated with the SacI-XhoI-cut vector pSUN3 (Figure 5A, construct map). In Figure 5A, fragment *d35S* comprises the duplicated 35S promoter (747 bp), fragment *rbcS* the rbcS transit peptide from pea (204 bp), fragment *KETO2* (1027 bp) the entire primary sequence encoding the

Haematococcus pluvialis ketolase, fragment *term* (761 bp) the CaMV polyadenylation signal.

- To prepare the expression vector pS3KETO3, the 2.7 kb SacI/XhoI fragment from pJKETO3 was ligated with the SacI-XhoI-cut vector pSUN3 (Figure 6, construct map). In Figure 6, fragment *d35S* comprises the duplicated 35S promoter (747 bp), fragment *rbcS* the rbcS transit peptide from pea (204 bp), fragment *KETO3* (985 bp) the primary sequence encoding the *Haematococcus pluvialis* ketolase which has been truncated by 14 N-terminal amino acids, fragment *term* (761 bp) the CaMV polyadenylation signal.

- To prepare the expression vector pS3KETO4, the 2.8 kb SacI/XhoI fragment from pJKETO4 was ligated with the SacI-XhoI-cut vector pSUN3 (Figure 7, construct map). In Figure 7, fragment *d35S* comprises the duplicated 35S promoter (747 bp), fragment *rbcS* the rbcS transit peptide from pea (204 bp), fragment KETO4 (1038 bp) the entire primary sequence encoding the *Haematococcus pluvialis* ketolase with C-terminal myc-tag, fragment *term* (761 bp) the CaMV polyadenylation signal.

An expression cassette for the agrobacterium-mediated transformation of the ketolase from *Haematococcus pluvialis* in Tagetes erecta was prepared using the binary vector pSUN5 (WO02/00900).

- To prepare the Tagetes expression vector pS5KETO2, the 2.8 kb SacI/XhoI fragment from pJKETO2 was ligated with the SacI-XhoI-cut vector pSUN5 (Figure 5B, construct map). In Figure 5B, fragment *d35S* comprises the duplicated 35S promoter (747 bp), fragment *rbcS* the rbcS transit peptide from pea (204 bp), fragment *KETO2* (1027 bp) the entire primary sequence encoding the *Haematococcus pluvialis* ketolase, fragment *term* (761 bp) the caMV polyadenylation signal.

Example 5A:
Preparation of expression vectors for the flower-specific expression of the *Haematococcus pluvialis* ketolase in *Lycopersicon esculentum* and *Tagetes erecta*.

The ketolase from *Haematococcus pluvialis* was expressed in *L. esculentum* and *Tagetes erecta* using the transit peptide rbcS from pea (Anderson et al. 1986, Biochem J. 240:709-715). The expression was under the control of a modified version AP3P of the flower-specific promoter AP3 of *Arabidopsis thaliana*

(AL132971: nucleotide region 9298 to 10200; Hill et al. (1998) Development 125: 1711-1721).

The DNA fragment which comprises the AP3 promoter region -902 to +15 from *Arabidopsis thaliana* was prepared by means of PCR using genomic DNA (isolated from *Arabidopsis thaliana* by standard methods) and the primers PR7 (SEQ ID NO: 33) and PR10 (SEQ ID NO: 36).

The PCR conditions were as follows:

The PCR for the amplification of the DNA which comprises the AP3 promoter fragment (-902 to +15) was carried out in 50 µl of reaction mixture comprising:

- 100 ng of genomic DNA from *A. thaliana*
- 0.25 mM dNTPs
- 0.2 mM PR7 (SEQ ID NO: 33)
- 0.2 mM PR10 (SEQ ID NO: 36)
- 5 µl 10X PCR buffer (Stratagene)
- 0.25 µl Pfu polymerase (Stratagene)
- 28.8 µl distilled water.

The PCR was carried out under the following cycling conditions:

1X	94°C	2 minutes
35X	94°C	1 minute
	50°C	1 minute
	72°C	1 minute
1X	72°C	10 minutes

The 922 bp amplificate was cloned into the PCR cloning vector pCR 2.1 (Invitrogen) using standard methods, giving rise to the plasmid pTAP3.

Sequencing the clone pTAP3 confirms a sequence which differs from the published AP3 sequence (AL132971, nucleotide region 9298 to 10200) only by one insertion (one G in position 9765 of the sequence AL132971) and one base substitution (one G instead of one A in position 9726 of the sequence AL132971). These nucleotide differences were reproduced in an independent amplification experiment and thus represent the actual nucleotide sequence in the *Arabidopsis thaliana* plants used.

The modified version AP3P was prepared by means of recombinant PCR using the plasmid pTAP3. The region 10200 to 9771 was amplified using the primers PR7 (SEQ ID NO: 33) and PR9 (SEQ ID

NO: 35) (amplificate A7/9), and the region 9526 to 9285 was amplified using PR8 (SEQ ID NO: 34) and PR10 (SEQ ID NO: 36) (amplificate A8/10).

The PCR conditions were as follows:

The PCR reactions for the amplification of the DNA fragments which comprise the region 10200-9771 and the region 9526 to 9285 of the AP3 promoter were carried out in 50-µl batches of reaction mixture comprising:

- 100 ng AP3 amplificate (described above)
- 0.25 mM dNTPs
- 0.2 mM sense primer (PR7 SEQ ID NO: 33 and PR8 SEQ ID NO: 34, respectively)
- 0.2 mM antisense primer (PR9 SEQ ID NO: 35 and PR10 SEQ ID NO: 36, respectively)
- 5 µl 10X PCR buffer (Stratagene)
- 0.25 µl Pfu Taq polymerase (Stratagene)
- 28.8 µl distilled water.

The PCR was carried out under the following cycling conditions:

1X	94°C	2 minutes
35X	94°C	1 minute
	50°C	1 minute
	72°C	1 minute
1X	72°C	10 minutes

The recombinant PCR comprises annealing of the amplificates A7/9 and A8/10, which overlap over a sequence of 25 nucleotides, complementation to give a double strand, and subsequent amplification. This gives rise to a modified version of the AP3 promoter, viz. AP3P, in which the positions 9670 to 9526 are deleted. Denaturation (5 minutes at 95°C) and annealing (slow cooling at room temperature to 40°C) of the two amplificates A7/9 and A8/10 were carried out in 17.6 µl of reaction mixture comprising:

- 0.5 µg A7/9 amplificate
- 0.25 µg A8/10 amplificate

Filling in the 3' ends (30 minutes at 30°C) was carried out in 20 µl of reaction mixture comprising:

- 17.6 µg A7/9 and A8/10 annealing reaction (prepared as described above)

- 50 µM dNTPs
- 2 µl 1X Klenow buffer
- 2U Klenow enzyme

The nucleic acid encoding the modified promoter version AP3P was amplified by means of PCR using a sense-specific primer (PR7 SEQ ID NO: 33) and an antisense-specific primer (PR10 SEQ ID NO: 36).

The PCR conditions were as follows:

The PCR for the amplification of the AP3P fragment was carried out in 50 µl of reaction mixture comprising:

- 1 µl annealing reaction (prepared as described above)
- 0.25 mM dNTPs
- 0.2 mM PR7 (SEQ ID NO: 33)
- 0.2 mM PR10 (SEQ ID NO: 36)
- 5 µl 10X PCR buffer (Stratagene)
- 0.25 µl Pfu Taq polymerase (Stratagene)
- 28.8 µl distilled water.

The PCR was carried out under the following cycling conditions:

1X	94°C	2 minutes
35X	94°C	1 minute
	50°C	1 minute
	72°C	1 minute
1X	72°C	10 minutes

The PCR amplification with SEQ ID NO: 33 and SEQ ID NO: 36 resulted in a 778 bp fragment which encodes the modified promoter version AP3P. The amplificate was cloned into the cloning vector pCR2.1 (Invitrogen). Sequencing reactions with the primers T7 and M13 confirmed a sequence with identity to the sequence AL132971, region 10200 to 9298, with the internal region 9285 to 9526 having been deleted. This clone was therefore used for cloning into the expression vector pJIT117 (Guerineau et al. 1988, Nucl. Acids Res. 16: 11380).

Cloning was carried out by isolating the 771 bp SacI/HindIII fragment from pTAP3P and ligation into the SacI/HindIII-cut vector pJIT117. The clone which comprises the promoter AP3P instead of the original promoter d35S is named pJAP3P.

To prepare an expression cassette pJAP3PKETO2, the 1027 bp SpHI fragment KETO2 (described in Example 1) was cloned into the SpHI-cut vector pJAP3P. The clone which comprises the fragment

KETO2 in the correct orientation as N-terminal fusion with the rbcS transit peptide is named pJAP3PKETO2.

To prepare an expression cassette pJAP3PKETO4, the 1032 bp SpHI/EcoRI fragment KETO4 (described in Example 3) was cloned into the SpHI/EcoRI-cut vector pJAP3P. The clone which comprises the fragment KETO4 in the correct orientation as N-terminal fusion with the rbcS transit peptide is named pJAP3PKETO4.

An expression cassette for the agrobacterium-mediated transformation of the AP3P-controlled ketolase from *Haematococcus pluvialis* in *L. esculentum* was prepared using the binary vector pSUN3 (WO02/00900).

- To prepare the expression vector pS3AP3PKETO2, the 2.8 kb SacI/XhoI fragment from pJAP3PKETO2 was ligated with the SacI/XhoI-cut vector pSUN3 (Figure 8A, construct map). In Figure 8A, fragment *AP3P* comprises the modified AP3P promoter (771 bp), fragment *rbcS* the rbcS transit peptide from pea (204 bp), fragment *KETO2* (1027 bp) the entire primary sequence encoding the *Haematococcus pluvialis* ketolase, fragment *term* (761 bp) the CaMV polyadenylation signal.

- To prepare the expression vector pS3AP3PKETO4, the 2.8 kb SacI/XhoI fragment from pJAP3PKETO4 was ligated with the SacI/XhoI-cut vector pSUN3 (Figure 9, construct map). In Figure 9, fragment *AP3P* comprises the modified AP3P promoter (771 bp), fragment *rbcS* the rbcS transit peptide from pea (204 bp), fragment KETO4 (1038 bp) the entire primary sequence encoding the *Haematococcus pluvialis* ketolase with C-terminal myc-tag, fragment *term* (761 bp) the CaMV polyadenylation signal.

An expression vector for the agrobacterium-mediated transformation of the AP3P-controlled ketolase from *Haematococcus pluvialis* in *Tagetes erecta* was prepared using the binary vector pSUN5 (WO02/00900).

To prepare the expression vector pS5AP3PKETO2, the 2.8 kb SacI/XhoI fragment from pJAP3PKETO2 was ligated with the SacI/XhoI-cut vector pSUN5 (Figure 8B, construct map). In Figure 8B, fragment *AP3P* comprises the modified AP3P promoter (771 bp), fragment *rbcS* the rbcS transit peptide from pea (204 bp), fragment *KETO2* (1027 bp) the entire primary sequence encoding the *Haematococcus pluvialis* ketolase, fragment *term* (761 bp) the CaMV polyadenylation signal.

Example 5B:
Amplification of a chimeric cDNA which comprises the ketolase from *Haematococcus pluvialis* Flotow em. Wille with a heterologous 5'-untranslated region (5'-UTR), and preparation of an expression vector for the flower-specific expression of the *Haematococcus pluvialis* ketolase without the use of a heterologous transit peptide in *Lycopersicon esculentum*.

The cDNA which comprises the ketolase from *Haematococcus pluvialis* (strain 192.80) following a heterologous "5'-untranslated region" (5'-UTR) was generated by means of PCR.

The nucleic acid encoding a ketolase from *Haematococcus pluvialis* (strain 192.80) with a "5'-untranslated region" (5'-UTR) was amplified by means of polymerase chain reaction (PCR) from the plasmid pGKET02 using a sense-specific primer (PR142 SEQ ID NO: 78) and an antisense-specific primer.

The PCR conditions were the following:

The PCR for the amplification of the fragment which not only encodes a ketolase protein, but also comprises a heterologous 5'-UTR region, was carried out in 50 µl of reaction mixture comprising:

- 10 ng of the plasmid pGKET02 (described in Example 1)
- 0.25 mM dNTPs
- 0.2 mM PR1 (SEQ ID NO: 29)
- 0.2 mM PR142 (SEQ ID NO: 78)
- 5 µl 10X PCR buffer (TAKARA)
- 0.25 µl R Taq polymerase (TAKARA)
- 25.8 µl distilled water.

The PCR was carried out under the following cycling conditions:

1X	94°C	2 minutes
35X	94°C	1 minute
	53°C	2 minutes
	72°C	3 minutes
1X	72°C	10 minutes

The PCR amplification with PR1 and PR142 resulted in a 1.1 kb fragment which comprises a heterologous 5'-UTR region followed by the coding region for a ketolase (SEQ ID NO: 79).

The amplificate was cloned into the PCR cloning vector pCR2.1 (Invitrogen) using standard methods. Sequencing reactions the resulting clone pTA-KETO5 with the primers T7 and M13 confirmed a sequence (SEQ ID NO: 79) which [apart from the 5' terminus, which is identical to pJIT117 (Guerineau et al. 1988, Nucl. Acids Res. 16: 11380)], is identical to the sequence SEQ ID NO: 22. This clone was therefore used for cloning into the expression vector pJAP3PKETO2 (Example 5A).

Cloning was effected by isolating the 0.3 kb HindIII fragment from pTA-KETO5 and ligation into the HindIII-cut vector pJAP3PKETO2. The clone, which comprises the AP3P promoter followed by the 5'-UTR from pJIT117 and the complete coding sequence for the *Haematococcus pluvialis* ketolase is named pJAP3PKETO5.

Expression of the ketolase from *Haematococcus pluvialis* in *L. esculentum* was under the control of the promoter AP 3P (see Example 5A) and the 5'-UTR from pJIT117. An expression cassette for the agrobacterium-mediated transformation of the ketolase from *Haematococcus pluvialis* in *L. esculentum* was prepared using the binary vector pSUN3 (WO 02/009.00).

To prepare the expression vector pS3AP3PKETO5, the 2.8 kb SacI/XhoI fragment from pJAP3PKETO5 was ligated with the SacI/XhoI-cut vector pSUN3 (Figure 21, construct map). In Figure 21, fragment *AP3P* comprises the AP3P promoter (747 bp), fragment *5'-UTR* the 5'-UTR sequence from pJIT117 (30 bp), fragment *KETO5* (1.0 kb) the entire primary sequence encoding the *Haematococcus pluvialis* ketolase, fragment *term* (761 bp) the CaMV polyadenylation signal.

Example 6:
Generation and analysis of transgenic Lycopersicon esculentum plants

Tomato plants were transformed and regenerated by the published method of Ling and coworkers (Plant Cell Reports (1998), 17:843-847). A higher kanamycine concentration (100 mg/l) was used for the selection for the variety Microtom.

The starting explants for the transformation were cotyledons and hypocotyls of seven- to ten-day old seedlings of the line Microtom. The culture medium of Murashige and Skoog (1962: Murashige and Skoog, 1962, Physiol. Plant 15, 473-) supplemented with 2% sucrose, pH 6.1, was used for the germination. Germination took place at 21°C at a low light level (20 to 100

µE). After seven to ten days, the cotyledons were divided horizontally and the hypocotyls were cut into segments 5 to 10 mm in length and placed on the medium MSBN (MS, pH 6.1, 3% sucrose, + 1 mg/l BAP, 0.1 mg/l NAA) which had been charged on the day before with tomato cells grown in suspension culture. The tomato cells were covered with sterile paper filters in such a way that there were no air bubbles. The explants were precultured on the above-described medium for three to five days. Cells of the strain Agrobacterium tumefaciens LBA4404 were transformed individually with the plasmids pS3KETO2, pS3KETO3, pS3AP3PKETO5 and pS3AP3KETO2, respectively. In each case one overnight culture of the individual Agrobacterium strains which had been transformed with the binary vectors pS3KETO2 and pS3KETO3, respectively, was grown in YEB medium with kanamycine (20 mg/l) at 28 degrees Celsius, and the cells were centrifuged. The bacterial pellet was resuspended in liquid MS medium (3% sucrose, pH 6.1) and brought to an optical density of 0.3 (at 600 nm). The precultured explants were transferred into the suspension and incubated for 30 minutes at room temperature with gentle shaking. Thereafter, the explants were dried with sterile paper filters and returned to their preculture medium for three days of coculture (21°C).

After the coculture, the explants were transferred to MSZ2 medium (MS pH 6.1 + 3% sucrose, 2 mg/l zeatin, 100 mg/l kanamycin, 160 mg/l Timentin) and stored under low light conditions (20 to 100 µE, ohotoperiod 16 h/8 h) at 21°C for the selective regeneration. The explants are transferred every two to three weeks until shoots form. Small shoots were separated from the explants and rooted on MS (pH 6.1 + 3% sucrose), 160 mg/l Timentin, 30 mg/l kanamycine, 0.1 mg/l IAA. Rooted plants were transferred to the greenhouse.

In accordance with the above-described transformation method, the following lines were obtained with the following expression constructs:

the following were obtained with pS3KETO2: cs13-8, cs13-24, cs13-30, cs13-40.

the following were obtained with pS3KETO3: cs14-2, cs14-3, cs14-9, cs14-19.

the following were obtained with pS3AP3PKETO2: cs16-15, cs16-34, cs16-35, cs16-40.

Table 1a shows the phenotype of the petals of the tomato plants which have been genetically modified in accordance with the invention. The analysis of the ketocarotenoids was carried out as described below.

Table 1a

Plant	Petal color	Astaxanthin	Adonixanthin
Control	yellow	no	no
Control	yellow	no	no
CS13-8	orange	yes	yes
CS13-24	orange	yes	yes
CS13-30	orange	yes	yes
CS13-40	orange	yes	yes
CS14-2	orange	yes	yes
CS14-3	orange	yes	yes
CS14-9	orange	yes	yes
CS14-19	orange	yes	yes
CS16-15	orange	yes	yes
CS 16-34	orange	yes	yes
CS 16-35	orange	yes	yes
CS 16-40	orange	yes	yes

The carotenoids were quantified by extracting the pigments in acetone, subjecting the carotenoid esters to enzymatic hydrolysis and separating the liberated carotenoids by means of HPLC. Experimental details and running conditions of the HPLC separations are described in detail in Example 9.

Table 1b shows the carotenoid profile in petals of transgenic tomato plants produced in accordance with the above-described examples, including the controls. Carotenoid concentrations are means of different lines and are shown as a percentage of the total carotenoid content.

Table 1b

Tomato	Viola-xan-thin	Antha-xanthin	Lutein	Zeax-anthin	Crypto-xanthin	Beta/ zeta-carotene	Asta-xan-thin	Adoni-xanthin	Adoni-rubin	3'-Hydr-oxyech-inenone
control	70.6	14	13.2	1	0.2	0.95				
CS16 plant		0.5	1	3.2	0.3	15.3	61	4.1	15.2	
Cs 13 plant			9.7	0.4	0.05	9	68	1.3	12.3	0.2

Example 7:
Generation of transgenic Tagetes plants

Tagetes seeds are sterilized and placed on germination medium (MS medium; Murashige and Skoog, Physiol. Plant. 15(1962), 473-497) pH 5.8, 2% sucrose). Germination takes place in a temperature/light/time inverval of 18 to 28°C/20-200 µE/3 to 16 weeks, but preferably at 21°C, 20 to 70 µE, for 4 to 8 weeks.

All leaves of the *in vitro* plants which have developed until this point in time are harvested and cut transversely to the central vein. The resulting leaf explants, which have a size of 10 to 60 mm^2, are stored during the preparation in liquid MS medium at room temperature for not more than 2 hours.

Any Agrobacterium tumefaciens strain, but preferably a supervirulent strain such as, for example, EHA105 with a suitable binary plasmid, which can carry a selection marker gene (preferably *bar* or *pat*) and one or more trait or reporter genes (for example pS5KETO2 and pS5AP3PKETO2) is grown overnight and used for the cocultivation with the leaf material. The bacterial strain can be grown as follows: a single colony of the strain in question is inoculated into YEB (0.1% yeast extract, 0.5% beef extract, 0.5% peptone, 0.5% sucrose, 0.5% magnesium sulfate x 7 H_2O) supplemented with 25 mg/l kanamycine and grown at 28°C for 16 to 20 hours. Thereafter, the bacterial suspension is harvested by centrifugation at 6000 g for 10 minutes and resuspended in liquid MS medium in such a way that an OD_{600} of approx. 0.1 to 0.8 developed. This suspension is used for the cocultivation with the leaf material.

Immediately before the cocultivation, the MS medium in which the leaves have been stored is replaced by the bacterial suspension. The leaflets were incubated in the agrobacterial suspension for 30 minutes with gentle shaking at room temperature. Thereafter, the infected explants are placed on an MS medium which comprises growth regulators, such as, for example 3 mg/l benzylaminopurine (BAP) and 1 mg/l indolylacetic acid (IAA) and which has been solidified with agar (for example 0.8% Plant Agar (Duchefa, NL). The orientation of the leaves on the medium is of no importance. The explants are cultured for 1 to 8 days, but preferably for 6 days; during this process, the following conditions can be applied: light intensity: 30 to 80 µMol/m^2 x sec, temperature: 22 to 24°C, ohotoperiod 16/8 hours. Thereafter, the cocultured explants are transferred to fresh MS medium, preferably one which comprises the same growth regulators, this second medium additionally comprising an antibiotic for suppressing the growth

of the bacteria. Timentin in a concentration of from 200 to 500 mg/l is highly suitable for this purpose. The second selective component employed is one which selects for successful transformation. Phosphinothricin in a concentration of from 1 to 5 mg/l selects highly efficiently, but other selective components in accordance with the method to be used are also feasible.

After in each case one to three weeks, the explants are transferred to fresh medium, until shoot primoidia and small shoots develop which are subsequently transferred to the same basal medium including Timentin and PPT or alternative components with growth regulators, viz. e.g. 0.5 mg/l indolylbutyric acid (IBA) and 0.5 mg/l gibberellic acid GA_3 for rooting. Rooted shoots can be transferred into the greenhouse.

The following advantageous modifications are possible in addition to the method described:

- Before the explants are infected with the bacteria, they can be preincubated for 1 to 12 days, preferably 3 to 4 days, on the above-described coculture medium. This is followed by infection, coculture and selective regeneration as described above.

- The pH value can be reduced to pH 5.2 for the regeneration (normally 5.8). This improves the control of the agrobacterial growth.

- The addition of $AgNO_3$ (3 to 10 mg/l) to the regeneration medium improves the state of the culture including the regeneration itself.

- Components which reduce phenol formation and which are known to the skilled worker such as, for example, citric acid, ascorbic acid, PVP and many others, have a positive effect on the culture.

- It is also possible to use liquid culture medium for all of the method. The culture can also be incubated on commercially available supports which are positioned on the liquid medium.

In accordance with the above-described transformation method, the following expression constructs gave the following lines:

for example, the following were obtained with pS5KETO2: cs18-1 and cs18-2; for example the following were obtained with pS5AP3PKETO2: cs19-1, cs19-2 and cs19-3.

Example 8
Characterization of the flowers of the transgenic plants

Example 8.1
Separation of carotenoid esters in petals of transgenic plants

General protocol:

The petals of the transgenic plants are crushed in liquid nitrogen and the petal powder (approximately 40 mg) is extracted with 100% acetone (three portions of 500 µl each). The solvent is evaporated and the carotenoids are resuspended in 100 to 200 µl of petroleum ether/acetone (5:1, v/v).

The carotenoids are separated in concentrated form by means of thin-layer chromatography (TLC) on Silica60 F254 plates (Merck) in an organic solvent (petroleum ether/acetone; 5:1) on the basis of their phobicity. Yellow (xanthophyll esters), red (ketocarotenoid esters) and orange bands (mixture of xanthophyll esters and ketocarotenoid esters) on the TLC are scraped off.

The silica-bound carotenoids are eluted three times with 500 µl of acetone, the solvent is evaporated, and the carotenoids are separated and identified by means of HPLC.

Using a C30 reversed-phase column it is possible to differentiate between mono- and diesters of the carotenoids. HPLC running conditions were virtually identical to a published method (Frazer et al.(2000), Plant Journal 24(4): 551-558). Identification of the carotenoids is possible on the basis of the UV-VIS spectra.

Petal material of the transgenic tomato plants CS13-8, cs13-24, cs13-30, cs13-40, cs14-2, cs14-3, cs14-9, cs14-19 was crushed and extracted with acetone. Extracted carotenoids were separated by means of TLC. Mono- and diesters of ketocarotenoids were detected in both lines; the monoesters were present in markedly lower concentrations than the diesters.

HPLC analyses revealed that diesters of xanthophylls (yellow band) and of the ketocarotenoids (red band) were present; the diester of the ketocarotenoids were present in approximately 10 times higher concentrations than the monoesters (Figure 10).

Petal material of the transgenic tomato plants cs16-15, cs16-34, cs16-35, cs16-40, which contain the AP3 promoter, was crushed in a pestle and mortar and extracted with acetone. Extracted carotenoids were separated by means of TLC. Monoesters of ketocarotenoids were not detected, or in extremely low concentrations only. Diesters of the ketocarotenoids were present in the same amount as in lines CS13 and CS14. Diesters of xanthophylls were little modified in terms of quantity in comparison with control plants.

Figure 9A shows a thin-layer chromatogram. The carotenoids from tomato petals were extracted with acetone and separated by means of thin-layer chromatography. Additional carotenoid bands [(1), (2) and (3)] were detected in the petals of transgenic tomato plants in comparison with control extracts.

Figure 10 shows an HPLC diagram. The additional carotenoid bands in the petals of transgenic tomato fruits (see (1–3) in Figure 9A) were extracted, eluted with acetone and analyzed with the aid of HPLC. (1) was identified as the monoester, (2) and (3) as diesters.

Example 9
Enzymatic hydrolysis of carotenoid esters and identification of the carotenoids

General protocol

Crushed petal material (50 to 100 mg fresh weight) is extracted with 100% acetone (three times 500 µl; shaking in each case for approximately 15 minutes). The solvent is evaporated. Carotenoids are subsequently taken up in 400 µl of acetone (absorption at 475 nm between 0.75 and 1.25) and treated for 5 minutes in an ultrasonic bath. The carotenoid extract is mixed with 300 µl of 50 mM Tris-HCl buffer (pH 7.0) and incubated for 5 to 10 minutes at 37°C. Thereafter, 100 to 200 µl of cholesterol esterase (stock solution: 6.8 units/ml of a Pseudomonas spec. cholesterol esterase) are added. After 8 to 12 hours, another 100 to 200 µl of enzyme are added; the esters are hydrolyzed within 24 hours by incubation at 37°C. After addition of 0.35 g Na_2SO_4 x $10H_2O$ and 500 µl of petroleum ether, the solution is mixed thoroughly and centrifuged (3 minutes; 4500 g). The petroleum ether phase is removed and mixed with 0.35 g of Na_2SO_4 x $10H_2O$ (anhydrous). Centrifugation for 1 minute at 10 000 g. The petroleum ether is evaporated and free carotenoids are taken up in 100 to 120 µl of acetone. Free carotenoids can be identified by means of HPLC and

C30 reversed-phase columns on the basis of their retention time and UV-VIS spectra.

Isolated ketocarotenoid esters (mono- and diesters) of lines CS13, CS14 and CS16 were hydrolyzed with cholesterol esterase and the liberated carotenoids were separated by means of HPLC. The carotenoids were identified on the basis of retention time and spectrum in comparison with carotenoid standards. Mono- and diesters comprise astaxanthin in high concentrations (90%) and adonixanthin in low concentrations (10%).
(See table and figures)

Figure 11 shows an HPLC diagram. The eluted esters from example 9 (figure 10) were hydrolyzed enzymatically and the hydrolysates were analyzed by means of HPLC. Both mono- and diesters comprise astaxanthin as main carotenoid and adonixanthin in low concentrations.

Example 10:
Preparation of a cloning vector for preparing inverted-repeat expression cassettes for the flower-specific expression of epsilon-cyclase dsRNAs in *Tagetes erecta*

The expression of inverted-repeat transcripts consisting of epsilon-cyclase fragments in *Tagetes erecta* was carried out under the control of a modified version AP3P of the flower-specific promoter AP3 from *Arabidopsis thaliana* (AL132971: nucleotide region 9298 to 10200; Hill et al. (1998) Development 125: 1711 to 1721).

In each case, the inverted-repeat transcript comprises a fragment in correct orientation (sense fragment) and a sequence-identical fragment in the opposite orientation (antisense fragment) which are linked with one another by a functional intron, the PIV2 intron of the potato ST-LH1 gene (Vancanneyt G. et al.(1990) Mol Gen Genet 220: 245-50).

The cDNA which encodes the *Arabidopsis thaliana* AP3 promoter (-902 to +15) was generated by means of PCR using genomic DNA (isolated from *Arabidopsis thaliana* by standard methods) and the primers PR7 (SEQ ID NO: 49) and PR10 (SEQ ID NO: 52).

The PCR conditions were as follows:

the PCR for the amplification of the DNA encoding the AP3 promoter fragment (-902 to +15) was carried out in 50 µl of reaction mixture comprising:

- 1 µl of genomic DNA from *A. thaliana* (diluted 1:100, prepared as described above)
- 0.25 mM dNTPs
- 0.2 mM PR7 (SEQ ID NO: 49)
- 0.2 mM PR10 (SEQ ID NO: 52)
- 5 µl 10X PCR buffer (Stratagene)
- 0.25 µl Pfu polymerase (Stratagene)
- 28.8 µl distilled water.

The PCR was carried out under the following cycling conditions:

1X	94°C	2 minutes
35X	94°C	1 minute
	50°C	1 minute
	72°C	1 minute
1X	72°C	10 minutes

The 922 bp amplificate was cloned into the PCR cloning vector pCR 2.1 (Invitrogen) using standard methods, giving rise to the plasmid pTAP3. Sequencing the clone pTAP3 confirms a sequence which differs from the published AP3 sequence (AL132971, nucleotide region 9298 to 10200) only by one insertion (one G in position 9765 of the sequence AL132971) and one base substitution (one G instead of one A in position 9726 of the sequence AL132971). These nucleotide differences were reproduced in an independent amplification experiment and thus represent the actual nucleotide sequence in the *Arabidopsis thaliana* plants used.

The modified version AP3P was prepared by means of recombinant PCR using the plasmid pTAP3. The region 10200 to 9771 was amplified using the primers PR7 (SEQ ID NO: 49) and PR9 (SEQ ID NO: 51) (amplificate A7/9), and the region 9526 to 9285 was amplified using PR8 (SEQ ID NO: 50) and PR10 (SEQ ID NO: 52) (amplificate A8/10).

The PCR conditions were as follows:

The PCR reactions for the amplification of the DNA fragments which encode the region 10200 to 9771 and the region 9526 to 9285 of the AP3 promoter were carried out in 50-µl batches of reaction mixture comprising:

- 100 ng AP3 amplificate (described above)
- 0.25 mM dNTPs
- 0.2 mM PR7 (SEQ ID NO: 49) and PR8 (SEQ ID NO: 50), respectively)
- 0.2 mM PR9 (SEQ ID NO: 51) and PR10 (SEQ ID NO: 52), respectively)
- 5 µl 10X PCR buffer (Stratagene)
- 0.25 µl Pfu Taq polymerase (Stratagene)
- 28.8 µl distilled water.

The PCR was carried out under the following cycling conditions:

1X	94°C	2 minutes
35X	94°C	1 minute
	50°C	2 minutes
	72°C	3 minutes
1X	72°C	10 minutes

The recombinant PCR comprises annealing of the amplificates A7/9 and A8/10, which overlap over a sequence of 25 nucleotides, complementation to give a double strand, and subsequent amplification. This gives rise to a modified version of the AP3 promoter, viz. AP3P, in which the positions 9670 to 9526 are deleted. Denaturation (5 minutes at 95°C) and annealing (slow cooling at room temperature to 40°C) of the two amplificates A7/9 and A8/10 were carried out in 17.6 µl of reaction mixture comprising:

- 0.5 µg A7/9
- 0.25 µg A8/10

Filling in the 3' ends (30 minutes at 30°C) was carried out in 20 µl of reaction mixture comprising:

- 17.6 µl A7/9 and A8/10 annealing reaction (prepared as described above)
- 50 µM dNTPs
- 2 µl 1X Klenow buffer
- 2U Klenow enzyme

The nucleic acid encoding the modified promoter version AP3P was amplified by means of PCR using a sense-specific primer (PR7 SEQ ID NO: 49) and an antisense-specific primer (PR10 SEQ ID NO: 52).

The PCR conditions were as follows:

The PCR for the amplification of the AP3P fragment is carried out in 50 µl of reaction mixture comprising:

- 1 µl annealing reaction (prepared as described above)
- 0.25 mM dNTPs
- 0.2 mM PR7 (SEQ ID NO: 49)
- 0.2 mM PR10 (SEQ ID NO: 52)
- 5 µl 10X PCR buffer (Stratagene)
- 0.25 µl Pfu Taq polymerase (Stratagene)
- 28.8 µl distilled water.

The PCR was carried out under the following cycling conditions:

1X	94°C	2 minutes
35X	94°C	1 minute
	50°C	1 minute
	72°C	1 minute
1X	72°C	10 minutes

The PCR amplification with PR7, SEQ ID NO: 49 and PR10, SEQ ID NO: 52 resulted in a 778 bp fragment which encodes the modified promoter version AP3P. The amplificate was cloned into the cloning vector pCR2.1 (Invitrogen). Sequencing reactions with the primers T7 and M13 confirmed a sequence with identity to the sequence AL132971, region 10200 to 9298, with the internal region 9285 to 9526 having been deleted. This clone was therefore used for cloning into the expression vector pJIT117 (Guerineau et al. 1988, Nucl. Acids Res. 16: 11380).

Cloning was carried out by isolating the 771 bp SacI/HindIII fragment from pTAP3P and ligation into the SacI/HindIII-cut vector pJIT117. The clone which comprises the promoter AP3P instead of the original promoter d35S is named pJAP3P.

A DNA fragment which comprises the PIV2 intron of the gene ST-LS1 was generated by means of PCR using plasmid DNA p35SGUS INT (Vancanneyt G. et al.(1990) Mol Gen Genet 220: 245-50) and the primers PR40 (Seq ID NO: 54) and PR41 (Seq ID NO: 55).

The PCR conditions were as follows:

The PCR for the amplification of the sequence of the intron PIV2 of the gene ST-LS1 was carried out in 50 µl of reaction mixture comprising:

- 1 µl p35SGUS INT
- 0.25 mM dNTPs

- 0.2 µM PR40 (SEQ ID NO: 54)
- 0.2 µM PR41 (SEQ ID NO: 55)
- 5 µl 10X PCR buffer (TAKARA)
- 0.25 µl R Taq polymerase (TAKARA)
- 28.8 µl distilled water.

The PCR was carried out under the following cycling conditions:

1X	94°C	2 minutes
35X	94°C	1 minute
	53°C	1 minute
	72°C	1 minute
1X	72°C	10 minutes

The PCR amplification with PR40 and PR41 resulted in a 206 bp fragment. The amplificate was cloned into the PCR cloning vector pBluntII (Invitrogen) using standard methods, giving rise to the clone pBluntII-40-41. Sequencing reactions of this clone with the primer SP6 confirmed a sequence which is identical to the corresponding sequence from the vector p35SGUS INT.

This clone was therefore used for cloning into the vector pJAP3P (described above).

Cloning was carried out by isolating the 206 bp SalI/BamHI fragment from pBluntII-40-41 and ligation with the SalI/BamHI-cut vector pJAP3P. The clone which comprises the intron PIV2 of the gene ST-LS1 in the correct orientation following the 3' terminus of the rbcs transit peptide is named pJAI1 and is suitable for the preparation of the expression cassettes for the flower-specific expression of inverted-repeat transcripts.

In Figure 12, fragment *AP3P* comprises the modified AP3P promoter (771 bp), fragment *rbcs* comprises the rbcS transit peptide from pea (204 bp), fragment *intron* the intron PIV2 of the potato gene ST-LS1, and fragment *term* (761 bp) the CaMV polyadenylation signal.

Example 11

Preparation of inverted-repeat expression cassettes for the flower-specific expression of epsilon-cyclase dsRNAs in *Tagetes erecta* (directed against the 5' region of the epsilon-cyclase cDNA)

The nucleic acid which comprises the 5'-terminal 435 bp region of the epsilon-cyclase cDNA (Genbank accession NO: AF251016) was amplified by means of polymerase chain reaction (PCR) from

Tagetes erecta cDNA using a sense-specific primer (PR42 SEQ ID NO: 56) and an antisense-specific primer (PR43 SEQ ID NO: 57). The 5'-terminal 435 bp region of the epsilon-cyclase cDNA from *Tagetes erecta* is composed of 138 bp 5'-untranslated sequence (5'-UTR) and 297 bp of the coding region which corresponds to the N terminus.

To prepare total RNA from Tagetes flowers, 100 mg of the frozen pulverized flowers were transferred to a reaction vessel and taken up in 0.8 ml of Trizol buffer (LifeTechnologies). The suspension was extracted with 0.2 ml of chloroform. After centrifugation for 15 minutes at 12 000 g, the aqueous supernatant was removed and transferred to a fresh reaction vessel and extracted with one volume of ethanol. The RNA was precipitated with one volume of isopropanol, washed with 75% of ethanol, and the pellet was dissolved in DEPC water (overnight incubation of water with 1/1000 volume of diethyl pyrocarbonate at room temperature, followed by autoclaving). The RNA concentration was determined photometrically. For the cDNA synthesis, 2.5 µg of total RNA were denatured for 10 minutes at 60°C, cooled on ice for 2 minutes, and transcribed into cDNA using a cDNA kit (Ready-to-go-you-prime-beads, Pharmacia Biotech) following the manufacturer's instructions and using an antisense-specific primer (PR17 SEQ ID NO: 53).

The conditions for the subsequent PCR reactions were as follows:

The PCR for the amplification of the PR42-PR43 DNA fragment which comprises the 5'-terminal 435 bp region of the epsilon-cyclase was carried out in 50 µl of reaction mixture comprising:

- 1 µl of cDNA (prepared as described above)
- 0.25 mM dNTPs
- 0.2 µM PR42 (SEQ ID NO: 56)
- 0.2 µM PR43 (SEQ ID NO: 57)
- 5 µl 10X PCR buffer (TAKARA)
- 0.25 µl R Taq polymerase (TAKARA)
- 28.8 µl distilled water.

The PCR for the amplification of the PR44-PR45 DNA fragment which comprises the 5'-terminal 435 bp region of the epsilon-cyclase was carried out in 50 µl of reaction mixture comprising:

- 1 µl of cDNA (prepared as described above)
- 0.25 mM dNTPs
- 0.2 µM PR44 (SEQ ID NO: 58)
- 0.2 µM PR45 (SEQ ID NO: 59)

- 5 µl 10X PCR buffer (TAKARA)
- 0.25 µl R Taq polymerase (TAKARA)
- 28.8 µl distilled water.

The PCR reactions were carried out under the following cycling conditions:

1X	94°C	2 minutes
35X	94°C	1 minute
	58°C	1 minute
	72°C	1 minute
1X	72°C	10 minutes

The PCR amplification with the primers PR42 and PR43 resulted in a 443 bp fragment, and the PCR amplification with the primers PR44 and PR45 resulted in a 444 bp fragment.

The two amplificates, viz. the PR42-PR43 (HindIII/SalI sense) fragment and the PR44-PR45 (EcoRI/BamHI antisense) fragment, were cloned into the PCR cloning vector pCR-BluntII (Invitrogen), using standard methods. Sequencing reactions with the primer SP6 confirmed in each case a sequence with identity to the published sequence AF251016 (SEQ ID NO: 38), apart from the restriction sites which had been introduced. These clones were therefore used for preparing an inverted-repeat construct in the cloning vector pJAI1 (see Example 10).

The first cloning step was carried out by isolating the 444 bp PR44-PR45 BamHI/EcoRI fragment from the cloning vector pCR-BluntII (Invitrogen) and ligation with the BamHI/EcoRI-cut vector pJAI1. The clone, which comprises the 5'-terminal region of the epsilon-cyclase in antisense orientation, is named pJAI2. The ligation gives rise to a transcriptional fusion between the antisense fragment of the 5'-terminal region of the epsilon-cyclase and the CaMV polyadenylation signal.

The second cloning step was carried out by isolating the 443 bp PR42-PR43 HindIII/SalI fragment from the cloning vector pCR-BluntII (Invitrogen) and ligation with the HindIII/SalI-cut vector pJAI2. The clone, which comprises the 435 bp 5'-terminal region of the epsilon-cyclase cDNA in sense orientation, is named pJAI3. The ligation gives rise to a transcriptional fusion between the AP3P and the sense fragment of the 5'-terminal region of the epsilon-cyclase.

To prepare an inverted-repeat expression cassette under the control of the CHRC promoter, a CHRC promoter fragment was amplified using genomic DNA from petunia (prepared by standard methods) and the primers PRCHRC5 (SEQ ID NO: 76) and PRCHRC3 (SEQ ID NO: 77). The amplificate was cloned into the cloning vector pCR2.1 (Invitrogen). Sequencing reactions of the resulting clone pCR2.1-CHRC with the primers M13 and T7 confirmed a sequence with identity to the sequence AF099501. This clone was therefore used for cloning into the expression vector pJAI3.

Cloning was effected by isolating the 1537 bp SacI/HindIII fragment from pCR2.1-CHRC and ligation into the SacI/HindIII-cut vector pJAI3. The clone which comprises the promoter CHRC instead of the original promoter AP3P is named pJCI3.

The expression vectors for the agrobacterium-mediated transformation of the AP3P-, or CHRC-, controlled inverted-repeat transcript into *Tagetes erecta* were prepared using the binary vector pSUN5 (WO02/00900).

To prepare the expression vector pS5AI3, the 2622 bp SacI/XhoI fragment from pJAI3 was ligated with the SacI/XhoI-cut vector pSUN5 (Figure 13, construct map).

In Figure 13, fragment *AP3P* comprises the modified AP3P promoter (771 bp), fragment *5sense* the 5'-region of the epsilon-cyclase from *Tagetes erecta* (435 bp) in sense orientation, fragment *intron* the intron PIV2 of the potato gene ST-LS1, fragment *5anti* the 5'-region of the epsilon-cyclase from *Tagetes erecta* (435 bp) in antisense orientation, and fragment *term* (761 bp) the CaMV polyadenylation signal.

To prepare the expression vector pS5CI3, the 3394 bp SacI/XhoI fragment from pJCI3 was ligated with the SacI/XhoI-cut vector pSUN5 (Figure 14, construct map).

In Figure 14, fragment *CHRC* comprises the promoter (1537 bp), fragment *5sense* the 5'-region of the epsilon-cyclase from *Tagetes erecta* (435 bp) in sense orientation, fragment *intron* the intron PIV2 of the potato gene ST-LS1, fragment *5anti* the 5'-region of the epsilon-cyclase from *Tagetes erecta* (435 bp) in antisense orientation, and fragment *term* (761 bp) the CaMV polyadenylation signal.

Example 12

Preparation of an inverted-repeat expression cassette for the flower-specific expression of epsilon-cyclase dsRNAs in *Tagetes*

erecta (directed against the 3′ region of the epsilon-cyclase cDNA)

The nucleic acid which comprises the 3′-terminal region (384 bp) of the epsilon-cyclase cDNA (Genbank accession NO: AF251016) was amplified by means of polymerase chain reaction (PCR) from *Tagetes erecta* cDNA using a sense-specific primer (PR46 SEQ ID NO: 60) and an antisense-specific primer (PR47 SEQ ID NO: 61). The 3′-terminal region (384 bp) of the epsilon-cyclase cDNA from *Tagetes erecta* is composed of 140 bp 3′-untranslated sequence (3′-UTR) and 244 bp of the coding region which corresponds to the C terminus.

The preparation of total RNA from Tagetes flowers was carried out as described in Example 11.

cDNA was synthesized as described in Example 11, using the antisense-specific primer PR17 (SEQ ID NO: 53).

The PCR reaction conditions were as follows:

The PCR for the amplification of the PR47-PR47 DNA fragment which comprises the 3′-terminal 384 bp region of the epsilon-cyclase was carried out in 50 µl of reaction mixture comprising:

- 1 µl of cDNA (prepared as described above)
- 0.25 mM dNTPs
- 0.2 µM PR46 (SEQ ID NO: 60)
- 0.2 µM PR47 (SEQ ID NO: 61)
- 5 µl 10X PCR buffer (TAKARA)
- 0.25 µl R Taq polymerase (TAKARA)
- 28.8 µl distilled water.

The PCR for the amplification of the PR48-PR49 DNA fragment which comprises the 5′-terminal 384 bp region of the epsilon-cyclase was carried out in 50 µl of reaction mixture comprising:

- 1 µl of cDNA (prepared as described above)
- 0.25 mM dNTPs
- 0.2 µM PR48 (SEQ ID NO: 62)
- 0.2 µM PR49 (SEQ ID NO: 63)
- 5 µl 10X PCR buffer (TAKARA)
- 0.25 µl R Taq polymerase (TAKARA)
- 28.8 µl distilled water.

The PCR reactions were carried out under the following cycling conditions:

1X	94°C	2 minutes
35X	94°C	1 minute
	58°C	1 minute
	72°C	1 minute
1X	72°C	10 minutes

The PCR amplification with SEQ ID NO: 60 and SEQ ID NO: 61 resulted in a 392 bp fragment, the PCR amplification with SEQ ID NO: 62 and SEQ ID NO: 63 resulted in a 396 bp fragment.

The two amplificates, viz. the PR46-PR47 fragment and the PR48-PR49 fragment, were cloned into the PCR cloning vector pCR-BluntII (Invitrogen) using standard methods. Sequencing reactions with the primer SP6 confirmed in each case a sequence with identity to the published sequence AF251016 (SEQ ID NO: 38), except for the restriction sites which had been introduced. These clones were therefore used for preparing an inverted-repeat construct in the cloning vector pJAI1 (see Example 10).

The first cloning step was carried out by isolating the 396 bp PR48-PR49 BamHI/EcoRI fragment from the cloning vector pCR-BluntII (Invitrogen) and ligation with the BamHI/EcoRI-cut vector pJAI1. The clone, which comprises the 3'-terminal region of the epsilon-cyclase in antisense orientation, is named pJAI4. The ligation gives rise to a transcriptional fusion between the antisense fragment of the 3'-terminal region of the epsilon-cyclase and the CaMV polyadenylation signal.

The second cloning step was carried out by isolating the 392 bp PR46-PR47 HindIII/SalI fragment from the cloning vector pCR-BluntII (Invitrogen) and ligation with the HindIII/SalI-cut vector pJAI4. The clone, which comprises the 392 bp 3'-terminal region of the epsilon-cyclase cDNA in sense orientation, is named pJAI5. The ligation gives rise to a transcriptional fusion between the AP3P and the sense fragment of the 3'-terminal region of the epsilon-cyclase.

An expression vector for the agrobacterium-mediated transformation of the AP3P-controlled inverted repeat transcript into Tagetes erecta was prepared using the binary vector pSUN5 (WO02/00900). To prepare the expression vector pS5AI5, the 2523 bp SacI/XhoI fragment from pJAI5 was ligated with the SacI/XhoI-cut vector pSUN5 (Figure 15, construct map).

In Figure 15, fragment *AP3P* comprises the modified AP3P promoter (771 bp), fragment *3sense* the 3'-region of the epsilon-cyclase from Tagetes erecta (435 bp) in sense orientation, fragment *intron* the intron IV2 of the potato gene ST-LS1, fragment *3anti* the 3'-region of the epsilon-cyclase from Tagetes erecta (435 bp) in antisense orientation, and fragment *term* (761 bp) the CaMV polyadenylation signal.

Example 13

Cloning the epsilon-cyclase promoter

A 199 bp fragment or the 312 bp fragment of the epsilon-cyclase promoter was isolated by two independent cloning strategies, inverted PCR (adapted from the method of Long et al. Proc. Natl. Acad. Sci USA 90: 10370) and TAIL-PCR (Liu Y-G. et al. (1995) Plant J. 8: 457-463) using genomic DNA (isolated from *Tagetes erecta*, line Orangenprinz, by standard method).

For the inverted PCR approach, 2 µg of genomic DNA were digested with EcoRV and RsaI in 25 µl of reaction mixture, subsequently diluted to 300 µl and religated with 3U of ligase at 16°C overnight. Using the primers PR50 (SEQ ID NO: 64) and PR51 (SEQ ID NO: 65), PCR amplification generated a fragment which, in each case in sense orientation, comprises 354 bp of the epsilon-cyclase cDNA (Genbank Accession AF251016), ligated with 300 bp of the epsilon-cyclase promoter, and 70 bp of the 5'-terminal region of the epsilon-cyclase cDNA (see Figure 16).

The conditions for the PCR reactions were as follows:

The PCR for the amplification of the PR50-PR51 DNA fragment which comprises inter alia, the 312 bp promoter fragment of the epsilon-cyclase was carried out in 50 µl of reaction mixture comprising:

- 1 µl of ligation mixture (prepared as described above)
- 0.25 mM dNTPs
- 0.2 µM PR50 (SEQ ID NO: 64)
- 0.2 µM PR51 (SEQ ID NO: 65)
- 5 µl 10X PCR buffer (TAKARA)
- 0.25 µl R Taq polymerase (TAKARA)
- 28.8 µl distilled water.

The PCR reactions were carried out under the following cycling conditions:

1X	94°C	2 minutes
35X	94°C	1 minute
	53°C	1 minute
	72°C	1 minute
1X	72°C	10 minutes

The PCR amplification with the primers PR50 and PR51 resulted in a 734 bp fragment which comprises, inter alia, the 312 bp promoter fragment of the epsilon-cyclase (Figure 16).

The amplificate was cloned into the PCR cloning vector pCR2.1 (Invitrogen) using standard methods. Sequencing reactions with the primers M13 and T7 gave the sequence SEQ ID NO: 45. This sequence was reproduced in an independent amplification experiment and thus represents the nucleotide sequence in the *Tagetes erecta* line used, Orangenprinz.

Three successive PCR reactions with in each case different gene-specific primers (nested primers) were carried out for the TAIL-PCR approach.

The TAIL1-PCR was carried out in 20 µl of reaction mixture comprising:

- 1 ng genomic DNA (prepared as described above)
- 0.2 mM of each dNTP
- 0.2 µM PR60 (SEQ ID NO: 66)
- 0.2 µM AD1 (SEQ ID NO: 69)
- 2 µl 10X PCR buffer (TAKARA)
- 0.5 U R Taq polymerase (TAKARA)
- made up to 20 µl with distilled water

  - here, AD1 was first a mixture of primers with the sequences (a/c/g/t)tcga(g/c)t(a/t)t(g/c)g(a/t)gtt dar.

The PCR reaction TAIL1 was carried out under the following cycling conditions:

1X 93°C: 1 minute, 95°C: 1 minute
5X 94°C: 30 seconds, 62°C: 1 minute, 72°C: 2.5 minutes
1X 94°C: 30 seconds, 25°C: 3 minutes, ramp to 72°C in 3 minutes, 72°C: 2.5 minutes
15X 94°C: 10 seconds, 68°C: 1 minute, 72°C: 2.5 minutes;
94°C: 10 seconds, 68°C: 1 minute, 72°C: 2.5 minutes;

94°C: 10 seconds, 29°C: 1 minute, 72°C: 2.5 minutes
1X 72°C: 5 minutes

The TAIL2-PCR was carried out in 21 µl of reaction mixture comprising:

- 1 µl of a 1:50 dilution of the TAIL1 reaction mixture (prepared as described above)
- 0.8 mM dNTP
- 0.2 µM PR61 (SEQ ID NO: 67)
- 0.2 µM AD1 (SEQ ID NO: 69)
- 2 µl 10X PCR buffer (TAKARA)
- 0.5 U R Taq polymerase (TAKARA)
- made up to 21 µl with distilled water

The PCR reaction TAIL2 was carried out under the following cycling conditions:

12X 94°C: 10 seconds, 64°C: 1 minute, 72°C: 2.5 minutes;
94°C: 10 seconds, 64°C: 1 minute, 72°C: 2.5 minutes;
94°C: 10 seconds, 29°C: 1 minute, 72°C: 2.5 minutes
1X 72°C: 5 minutes

The TAIL3-PCR was carried out in 100 µl of reaction mixture comprising:

- 1 µl of a 1:10 dilution of the TAIL2 reaction mixture (prepared as described above)
- 0.8 mM dNTP
- 0.2 µM PR63 (SEQ ID NO: 68)
- 0.2 µM AD1 (SEQ ID NO: 69)
- 10 µl 10X PCR buffer (TAKARA)
- 0.5 U R Taq polymerase (TAKARA)
- made up to 100 µl with distilled water

The PCR reaction TAIL3 was carried out under the following cycling conditions:

20X 94°C: 15 seconds, 29°C: 30 seconds, 72°C: 2 minutes
1X 72°C: 5 minutes

The PCR amplification with the primers PR63 and AD1 resulted in a 280 bp fragment which comprises, inter alia, the 199 bp promoter fragment of epsilon-cyclase (Figure 17).

The amplificate was cloned into the PCR cloning vector pCR2.1 (Invitrogen) using standard methods. Sequencing reactions with the primers M13 and T7 gave the sequence SEQ ID NO: 46. This sequence is identical to the sequence SEQ ID NO: 45, which had been isolated using the IPCR strategy, and thus represents the nucleotide sequence in the *Tagetes erecta* line used, Orangenprinz.

The pCR2.1 clone which comprises the 312 bp fragment (SEQ ID NO: 45) of the epsilon-cyclase promoter, which fragment had been isolated by IPCR strategy, is named pTA-ecycP and was used for the preparation of the IR construct.

Example 14

Preparation of an inverted-repeat expression cassette for the flower-specific expression of epsilon-cyclase dsRNAs in *Tagetes erecta* (directed against the promoter region of the epsilon-cyclase cDNA).

The expression of inverted-repeat transcripts consisting of epsilon-cyclase promoter fragments in *Tagetes erecta* was effected under the control of a modified version AP3P of the flower-specific promoter AP3 from *Arabidopsis* (see Example 10) or the flower-specific promoter CHRC (Genbank accession NO: AF099501). The inverted-repeat transcript comprises in each case one epsilon-cyclase promoter fragment in correct orientation (sense fragment) and one sequence-identical epsilon-cyclase promoter fragment in opposite orientation (antisense fragment) which are linked with one another by a functional intron (see Example 10).

The promoter fragments were generated by means of PCR using plasmid DNA (clone pTA-ecycP, see Example 13) and the primers PR124 (SEQ ID NO: 70) and PR126 (SEQ ID NO: 72) and, respectively, the primers PR125 (SEQ ID NO: 71) and PR127 (SEQ ID NO: 73).

The conditions for the PCR reactions were as follows:

The PCR for the amplification of the PR124-PR126 DNA fragment which comprises the epsilon-cyclase promoter fragment was carried out in 50 µl of reaction mixture comprising:

- 1 µl cDNA (prepared as described above)
- 0.25 mM dNTPs
- 0.2 µM PR124 (SEQ ID NO: 70)
- 0.2 µM PR126 (SEQ ID NO: 72)

- 5 µl 10X PCR buffer (TAKARA)
- 0.25 µl R Taq polymerase (TAKARA)
- 28.8 µl distilled water

The PCR for the amplification of the PR125-PR127 DNA fragment which comprises the epsilon-cyclase 312 bp promoter fragment was carried out in 50 µl of reaction mixture comprising:

- 1 µl cDNA (prepared as described above)
- 0.25 mM dNTPs
- 0.2 µM PR125 (SEQ ID NO: 71)
- 0.2 µM PR127 (SEQ ID NO: 73)
- 5 µl 10X PCR buffer (TAKARA)
- 0.25 µl R Taq polymerase (TAKARA)
- 28.8 µl distilled water

The PCR reactions were carried out under the following cycling conditions:

1X	94°C	2 minutes
35X	94°C	1 minute
	53°C	1 minute
	72°C	1 minute
1X	72°C	10 minutes

The PCR amplification with the primers PR124 and PR126 resulted in a 358 bp fragment, and PCR amplification with the primers PR125 and PR127 resulted in a 361 bp fragment.

The two amplificates, viz. the PR124-PR126 (HindIII/SalI sense) fragment and the PR125-PR127 (EcoRI/BamHI antisense) fragment, were cloned into the PCR cloning vector pCR-BluntII (Invitrogen) using standard methods. Sequencing reactions with the primer SP6 confirmed in each case a sequence which is identical to SEQ ID NO: 45, except for the restriction sites which have been introduced. These clones were therefore used for generating an inverted-repeat construct in the cloning vector pJAI1 (see Example 10).

The first cloning step was carried out by isolating the 358 bp PR124-PR126 HindIII/SalI fragment from the cloning vector pCR-BluntII (Invitrogen) and ligation with the BamHI/EcoRI-cut vector pJAI1. The clone comprising the epsilon-cyclase promoter fragment in sense orientation is named cs43. The sense fragment of the epsilon-cyclase promoter is inserted between the AP3P promoter and the intron by means of ligation.

The second cloning step was carried out by isolating the 361 bp PR125-PR127 BamHI/EcoRI fragment from the cloning vector pCR-BluntII (Invitrogen) and ligation with the BamHI/EcoRI-cut vector cs43. The clone comprising the epsilon-cyclase promoter fragment in antisense orientation is named cs44. Ligation gives a transcriptional fusion between the intron and the antisense fragment of the epsilon-cyclase promoter.

To generate an inverted-repeat expression cassette under the control of the CHRC promoter, a CHRC promoter fragment was amplified using genomic DNA from petunia (prepared by standard methods) and the primers PRCHRC3' (SEQ ID NO: 77) and PRCHRC5' (SEQ ID NO: 76). The amplificate was cloned into the cloning vector pCR2.1 (Invitrogen). Sequencing reactions of the resulting clone pCR2.1-CHRC with the primers M13 and T7 confirmed a sequence which was identical to the sequence AF099501. This clone was therefore used for cloning into the expression vector cs44.

Cloning was effected by isolating the 1537 bp SacI/HindIII fragment from pCR2.1-CHRC and ligation into the SacI/HindIII-cut vector cs44. The clone comprising the promoter CHRC instead of the original promoter AP3P is named cs45.

To prepare an inverted-repeat expression cassette under the control of two promoters, viz. the CHRC promoter and the AP3P promoter, the AP3P promoter was cloned into cs45 in antisense orientation onto the 3' terminus of the epsilon-cyclase antisense fragment. The AP3P promoter fragment from pJAI1 was amplified using the primers PR128 and PR129. The amplificate was cloned into the cloning vector pCR2.1 (Invitrogen). The sequencing reactions with the primers M13 and T7 confirmed a sequence which was identical to sequence SEQ ID NO: 28 (AL132971). This clone pCR2.1-AP3PSX was used for the preparation of an inverted-repeat expression cassette under the control of two promoters.

Cloning was effected by isolating the 771 bp SalI/XhoI fragment from pCR2.1-AP3PSX and ligation into the XhoI-cut vector cs45. The clone which comprises the promoter AP3P in antisense orientation 3' of the inverted repeat is named cs46.

The expression vectors for the Agrobacterium-mediated transformation of the AP3P-controlled inverted-repeat transcript in Tagetes erecta was prepared using the binary vector pSUN5 (WO02/00900).

To prepare the expression vector pS5AI7, the 1685 bp SacI/XhoI fragment from cs44 was ligated with the SacI/XhoI-cut vector pSUN5 (Figure 18, construct map). In Figure 18, fragment *AP3P* comprises the modified AP3P promoter (771 bp), fragment *P-sense* the 312 bp epsilon-cyclase promoter fragment in sense orientation, fragment *intron* the intron IV2 of the potato gene (ST-LS1), and fragment *P-anti* the 312 bp epsilon-cyclase promoter fragment in antisense orientation.

To prepare the expression vector pS5CI7, the 2445 bp SacI/XhoI fragment from cs45 was ligated with the SacI/XhoI-cut vector pSUN5 (Figure 19, construct map).

In Figure 19, fragment *CHRC* comprises the CHRC promoter (1537 bp), fragment *P-sense* the 312 bp epsilon-cyclase promoter fragment in sense orientation, fragment *intron* the intron IV2 of the potato gene ST-LS1, and fragment *P-anti* the 312 bp epsilon-cyclase promoter fragment in antisense orientation.

To prepare the expression vector pS5CAI7, the 3219 bp SacI/XhoI fragment from cs45 was ligated with the SacI/XhoI-cut vector pSUN5 (Figure 20, construct map).

In Figure 20, fragment *CHRC* comprises the CHRC promoter (1537 bp), fragment *P-sense* the 312 bp epsilon-cyclase promoter fragment in sense orientation, fragment *intron* the intron IV2 of the potato gene ST-LS1, fragment *P-anti* the 312 bp epsilon-cyclase promoter fragment in antisense orientation and fragment *AP3P* the 771 bp AP3P promoter fragment in antisense orientation.

Example 15
Generation of transgenic Tagetes plants with reduced ε-cyclase activity

Tagetes seeds are sterilized and placed on germination medium (MS medium; Murashige and Skoog, Physiol. Plant. 15(1962), 473-497, pH 5.8, 2% sucrose). Germination takes place under the conditions of a temperature/light/time interval of 18 to 28°C/20 to 200 µE/ 3 to 16 weeks, but preferably at 21°C, 20 to 70 µE, for 4 to 8 weeks.

All leaves of the *in vitro* plants which have developed until this point in time are harvested and cut transversely to the central vein. The resulting leaf explants, which have a size of 10 to 60 mm^2, are stored during the preparation in liquid MS medium at room temperature for not more than 2 hours.

The Agrobacterium tumefaciens strain EHA105 was transformed with the binary plasmid pS5AI3. The transformed A. tumefaciens strain EHA105 was grown overnight under the following conditions: a single colony was inoculated into YEB (0.1% yeast extract, 0.5% beef extract, 0.5% peptone, 0.5% sucrose, 0.5% magnesium sulfate x 7 H_2O) supplemented with 25 mg/l kanamycine and grown at 28°C for 16 to 20 hours. Thereafter, the bacterial suspension was harvested by centrifugation at 6000 g for 10 minutes and resuspended in liquid MS medium in such a way that an OD_{600} of approx. 0.1 to 0.8 developed. This suspension was used for the cocultivation with the leaf material.

Immediately before the cocultivation, the MS medium in which the leaves have been stored is replaced by the bacterial suspension. The leaflets were incubated in the agrobacterial suspension for 30 minutes with gentle shaking at room temperature. Thereafter, the infected explants are placed on an MS medium which comprises growth regulators, such as, for example 3 mg/l benzylaminopurine (BAP) and 1 mg/l indolylacetic acid (IAA) and which has been solidified with agar (for example 0.8% Plant Agar (Duchefa, NL). The orientation of the leaves on the medium is of no importance. The explants are cultured for 1 to 8 days, but preferably for 6 days; during this process, the following conditions can be applied: light intensity: 30 to 80 $\mu Mol/m^2$ x sec, temperature: 22 to 24°C, photoperiod of 16/8 hours. Thereafter, the cocultured explants are transferred to fresh MS medium, preferably one which comprises the same growth regulators, this second medium additionally comprising an antibiotic for suppressing the growth of the bacteria. Timentin in a concentration of from 200 to 500 mg/l is highly suitable for this purpose. The second selective component employed is one which selects for successful transformation. Phosphinothricin in a concentration of from 1 to 5 mg/l selects highly efficiently, but other selective components in accordance with the method to be used are also feasible.

After in each case one to three weeks, the explants are transferred to fresh medium, until shoot primordia and small shoots develop which are subsequently transferred to the same basal medium including Timentin and PPT or alternative components with growth regulators, viz. e.g. 0.5 mg/l indolylbutyric acid (IBA) and 0.5 mg/l gibberellic acid GA_3 for rooting. Rooted shoots can be transferred into the greenhouse.

The following advantageous modifications are possible in addition to the method described:

- Before the explants are infected with the bacteria, they can be preincubated for 1 to 12 days, preferably 3 to 4 days, on the above-described coculture medium. This is followed by infection, coculture and selective regeneration as described above.

- The pH value can be reduced to pH 5.2 for the regeneration (normally 5.8). This improves the control of the agrobacterial growth.

- The addition of $AgNO_3$ (3 to 10 mg/l) to the regeneration medium improves the state of the culture including the regeneration itself.

- Components which reduce phenol formation and which are known to the skilled worker such as, for example, citric acid, ascorbic acid, PVP and many others, have a positive effect on the culture.

- It is also possible to use liquid culture medium for all of the method. The culture can also be incubated on commercially available supports which are positioned on the liquid medium.

In accordance with the above-described transformation method, the following lines were obtained using the expression construct pS5AI3:

CS30-1, CS30-3 and CS30-4

Example 16:
Characterization of the transgenic Tagetes plants with reduced ε-cyclase activity

The petal material of the transgenic Tagetes erecta plants of Example 15 were crushed in liquid nitrogen, and the powder (approximately 250 to 500 mg) was extracted with 100% acetone (three 500 µl portions). The solvent was evaporated and the carotenoids were resuspended in 100 µl of acetone.

Using a C30 reversed-phase column it was possible to quantify the individual carotenoids. HPLC running conditions were virtually identical with a published method (Frazer et al. (2000), Plant Journal 24(4): 551-558). Identification of the carotenoids was possible on the basis of the UV-VIS spectra.

Table 2 shows the carotenoid profile in Tagetes petals of the transgenic Tagetes plants prepared in accordance with the above-described examples and of the control Tagetes plants. All carotenoid quantities are shown in [µg/g] fresh weight; changes in percent on the basis of the control plant are shown in brackets.

In comparison with the genetically non-modified control plant, the genetically modified plants with reduced epsilon-cyclase activity show a markedly increased content of carotenoids of the "β-carotene pathway", such as, for example, β-carotene and zeaxanthin, and a markedly reduced content of carotenoids of the "α-carotene pathway", such as, for example, lutein.

Table 2

Plant	Lutein	β-Carotene	Zeaxanthin	Violaxanthin	Total carotenoids
Control	260	4.8	2.7	36	304
CS 30-1	35 (-86%)	13 (+170%)	4.4 (+62%)	59 (+63%)	111 (-63%)
Control	456	6.4	6.9	58	527
CS 30-3	62 (-86%)	13 (+103%)	8.9 (+29%)	75 (+29%)	159 (-70%)
CS 30-4	68 (-85%)	9.1 (+42%)	5.7 (-17%)	61 (+5%)	144 (-73%)

Example 17:
Characterization of transgenic Tagetes plants which accumulate astaxanthin in petals

The petal material of the transgenic Tagetes erecta plants (of Example 7 with plasmid pS5AP3PKETO2) is crushed in liquid nitrogen, and the powder (approximately 30-100 mg) is extracted with 100% acetone (three 500 µl portions). The solvent is evaporated, and the carotenoids are resuspended in 30 µl of petroleum ether:acetone (ratio 5:1) and separated on a silica thin-layer plate. Tagetes plants with additional red carotenoid bands which do not occur in control plants were selected for preparative-analytical analyses. For analytical details, see Example 9.

The individual carotenoids are quantified by means of a C30 reversed-phase column. For analytical details, see Example 9.

Table 3 shows the carotenoid profile in Tagetes petals of the transgenic Tagetes plants prepared in accordance with the above-described examples and of control Tagetes plants.

Carotenoid concentrations are shown as percentages based on the total carotenoid content.

In comparison with the genetically non-modified control plants, the genetically modified plants which express a ketolase show an astaxanthin content.

Table 3: Percentage carotenoid concentrations in astaxanthin-synthetisizing Tagetes and in control plants

Tagetes plant	Anthera-xanthin	Lutein	Zea-xanthin	Crypto-xanthin	Beta/zeta-carotene	Asta-xanthin	Adonirubin	3'-Hydroxy-echinenone	3-Hydroxy-echinenone
control	1.5	93.6	1.2	0.3	3.8				
cs19-3	1.3	94.2	1.1	0.3	3.5	0.1		0.05	0.01
CHRC:: Ketolase	1.3-1.5	93.5-94.4	0.9-1.7	0.01-0.02	2-3.1	0.3-0.9	0.03-0.2	0.2	0-0.01
DFR-A:: Ketolase	4.5	91.8	1.1		2.4	0.2	0.02	0.07	

Example 18:

Characterization of transgenic Tagetes plants which have a reduced lutein concentration and which accumulate astaxanthin in petals

Tagetes plants which, as the result of the use of the AP3P promoter and the *Haematococcus* ketolase, synthesize astaxanthin in petals (see experimental details re pS5AP3PKETO2 in Example 5A) and Tagetes plants which, as the result of the use of the RNAi construct pS5AI3 (see Example 11, Figure 13), accumulate smaller amounts of lutein by means of the AP3P promoter were crossed. Seeds were germinated, and the progeny was subjected to molecular-biological and biochemical analysis.

The presence of the expression cassettes in question is studied by genomic PCR. To this end, young leaf material is harvested and used for isolating genomic DNA.

The integrity of the DNA preparation is checked by amplifying an endogenous gene segment from the Tagetes ε-cyclase which is not present in any of the expression cassettes by means of forward primer PR29 (PR29: 5'-cccattctcataggtcgtgc-3') and reverse primer PR78 (PR78: 5'-gcaagcctgcatggaattgtg-3'). In the case of intact genomic DNA, this PCR reaction results in a 0.6 kb fragment.

The ketolase expression cassette can be detected by genomic PCR by means of forward primer PR7 (PR7: 5'-gagctcactcactgatttccattgcttg-3') and reverse primer PR185 (PR185: 5'-cattaagctgcctgtttctca-3'). In the presence, but not in the absence, of the ketolase expression cassettes, this PCR reaction leads to the production of a 0.4 kb fragment.

Die ε-cyclase downregulation cassette can be detected by genomic PCR by means of forward primer PR7 and reverse primer PR41 (PR41: 5'-ggatccggtgatacctgcacatcaac-3'). In the presence, but not in the absence, of the ε-cyclase downregulation cassette, this PCR reaction leads to the production of a 1.4 kb fragment.

The conditions of the PCR reactions are as follows:

The PCR for the amplification of the fragments described is carried out in each case in 50 µl of reaction mixture comprising:

- 1 µl cDNA (prepared as described above)
- 0.25 mM dNTPs
- 0.2 µM of the respective forward primer
- 0.2 µM of the respective reverse primer
- 5 µl 10X PCR buffer (TAKARA)
- 0.25 µl R Taq polymerase (TAKARA)
- 28.8 µl distilled water

The PCR reactions are carried out under the following cyclic conditions and subsequently analyzed by agarose gel electrophoresis.

1X	94°C	2 minutes
35X	94°C	1 minute
	58°C	1 minute
	72°C	1 minute
1X	72°C	10 minutes

For the biochemical screening, the flower material of the Tagetes erecta plants is crushed in liquid nitrogen, and the powder (approximately 30 to 100 mg) is extracted with 100% acetone (three 500 µl portions). The solvent is evaporated, and the carotenoids are resuspended in 30 µl of petroleum ether:acetone (ratio 5:1) and separated on a silica thin-layer plate. Tagetes plants which show red carotenoid bands, which allow the conclusion that astaxanthin has been synthesized, and simultaneously less intensive lutein ester bands (one of the most mobile bands near the front of the mobile phase) were selected for preparative-analytical analyses. The individual carotenoids are quantified by hydrolyzing the esters by lipase treatment and separating the carotenoid mixture by means of HPLC. For analytical details, see Example 9.

Table 4 shows the carotenoid profile in Tagetes petals of the transgenic Tagetes plants produced by crossing in accordance with the above-described examples. Carotenoid concentrations are percentages based on the total carotenoid content.

In comparison with the genetically non-modified control plants, the genetically modified plants with reduced epsilon-cyclase activity and simultaneous synthesis of astaxanthin show i) a markedly increased content of carotenoids of the "β-carotene pathway", such as, for example, β-carotene and zeaxanthin, ii) a markedly reduced content of carotenoids of the "α-carotene pathway", such as, for example, lutein, and iii) accumulation of astaxanthin.

Table 4: Percentage carotenoid concentrations in transgenic Tagetes and control plants

Tagetes plant	Violaxanthin	Antheraxanthin	Lutein	Zeaxanthin	Cryptoxanthin	Beta/zeta-carotene	Astaxanthin	3'-Hydroxyechinenone	3-Hydroxyechinenone
control		1.5	93.6	1.2	0.3	3.8			
T109-26	0.6	2.1	65.9	10.4	0.1	19.9	0.3	0.7	0.08
T105-8		3	67.3	8.2	0.1	20.7	0.05	0.4	
T112-5		2.1	48.4	43.6	0.08	5.3	0.05	0.5	

Example 19:
Amplification of a DNA which encodes the entire primary sequence of the NP196-ketolase from *Nostoc punctiforme ATCC 29133*

The DNA which encodes the NP196-ketolase from *Nostoc punctiforme ATCC 29133* was amplified from *Nostoc punctiforme ATCC 29133* (strain of the "American Type Culture Collection") by means of PCR.

To prepare genomic DNA from a suspension culture of *Nostoc punctiforme ATCC 29133* which had been grown for 1 week under continuous light with constant shaking (150 rpm) at 25°C in *BG 11* medium (1.5 g/l $NaNO_3$, 0.04 g/l $K_2PO_4x3H_2O$, 0.075 g/l $MgSO_4xH_2O$, 0.036 g/l $CaCl_2x2H_2O$, 0.006 g/l citric acid, 0.006 g/l ferric ammonium citrate, 0.001 g/l EDTA disodium magnesium, 0.04 g/l Na_2CO_3, 1 ml Trace Metal Mix "A5+Co", 2.86 g/l H_3BO_3, 1.81 g/l $MnCl_2x4H_2O$, 0,222 g/l $ZnSO_4x7H_2O$, 0.39 g/l $NaMoO_4X2H_2O$, 0.079 g/l $CuSO_4x5H_2O$, 0.0494 g/l $Co(NO_3)_2x6H_2O$), the cells were harvested by centrifugation, frozen in liquid nitrogen and ground to a powder in a mortar.

Protocol for the DNA isolation from *Nostoc punctiforme ATCC 29133*:

The bacterial cells were pelleted from a 10 ml liquid culture by centrifugation for 10 minutes at 8000 rpm. Thereafter, the bacterial cells were comminuted and ground in liquid nitrogen using a pestle and mortar. The cell material was resuspended in 1 ml of 10mM Tris-HCl (pH 7.5) and transferred to an Eppendorf reaction vessel (volume 2 ml). After addition of 100 µl of Proteinase K (concentration: 20 mg/ml), the cell suspension was incubated for 3 hours at 37°C. Thereafter, the suspension was extracted with 500 µl of phenol. After centrifugation for 5 minutes at 13 000 rpm, the aqueous top phase was transferred to a fresh 2 ml Eppendorf reaction vessel. The phenol extraction was repeated 3 times. The DNA was precipitated by addition of 1/10 volume 3 M sodium acetate (pH 5.2) and 0.6 volume isopropanol and subsequently washed with 70% ethanol. The DNA pellet was dried at room temperature, taken up in 25 µl of water and dissolved with heating at 65°C.

The nucleic acid encoding a ketolase from *Nostoc punctiforme ATCC 29133* was amplified from *Nostoc punctiforme ATCC 29133* by means of polymerase chain reaction (PCR) using a sense-specific primer (NP196-1, SEQ ID No. 129) and an antisense-specific primer (NP196-2 SEQ ID No. 130).

The PCR conditions were as follows:

The PCR for the amplification of the DNA which encodes a ketolase protein consisting of the entire primary sequence was carried out in 50 µl of reaction mixture comprising:

- 1 µl of a *Nostoc punctiforme ATCC 29133* DNA (prepared as described above)
- 0.25 mM dNTPs
- 0.2 mM NP196-1 (SEQ ID No. 129)
- 0.2 mM NP196-2 (SEQ ID No. 130)
- 5 µl 10X PCR buffer (TAKARA)
- 0.25 µl R Taq polymerase (TAKARA)
- 25.8 µl distilled water

The PCR was carried out under the following cyclic conditions:

1X	94°C	2 minutes
35X	94°C	1 minute
	55°C	1 minute
	72°C	3 minutes
1X	72°C	10 minutes

The PCR amplification with SEQ ID No. 129 and SEQ ID No. 130 resulted in a 792 bp fragment which encodes a protein consisting of the entire primary sequence (NP196, SEQ ID No. 131). The amplificate was cloned into the PCR cloning vector pCR 2.1 (Invitrogen) using standard methods, giving rise to the clone pNP196.

Sequencing of the clone pNP196 with the M13F and the M13R primer verified a sequence which is identical to the DNA sequence of 140.571-139.810 of the database entry NZ_AABC01000196 (with inverse orientation relative to the published database entry), with the exception that G in position 140.571 was replaced by A in order to generate a standard ATG start codon. This nucleotide sequence was reproduced in an independent amplification experiment and thus represents the nucleotide sequence in the *Nostoc punctiforme ATCC 29133* used.

This clone pNP196 was therefore used for cloning into the expression vector pJIT117 (Guerineau et al. 1988, Nucl. Acids Res. 16: 11380).

pJIT117 was modified by replacing the 35S terminator by the OCS terminator (octopine synthase) of the Ti plasmid pTi15955 of Agrobacterium tumefaciens (database entry X00493, position 12.541-12.350, Gielen et al. (1984) EMBO J. 3 835-846).

The DNA fragment which comprises the OCS terminator region was prepared by means of PCR using the plasmid pHELLSGATE (database entry AJ311874, Wesley et al. (2001) Plant J. 27 581-590, isolated from *E.coli* by standard methods) and the primers OCS-1 (SEQ ID No. 133) and OCS-2 (SEQ ID No. 134).

The PCR conditions were as follows:

The PCR for the amplification of the DNA which comprises the octopine synthase (OCS) terminator region (SEQ ID No. 135) was carried out in 50 µl of reaction mixture comprising:

- 100 ng pHELLSGATE plasmid DNA

- 0.25 mM dNTPs
- 0.2 mM OCS-1 (SEQ ID No. 133)
- 0.2 mM OCS-2 (SEQ ID No. 134)
- 5 µl 10X PCR buffer (Stratagene)
- 0.25 µl Pfu polymerase (Stratagene)
- 28.8 µl distilled water

The PCR was carried out under the following cycling conditions:

1X	94°C	2 minutes
35X	94°C	1 minute
	50°C	1 minute
	72°C	1 minute
1X	72°C	10 minutes

The 210 bp amplificate was cloned into the PCR cloning vector pCR 2.1 (Invitrogen) using standard conditions, giving rise to the plasmid pOCS.

Sequencing of the clone pOCS verified a sequence which agrees with a sequence segment on the Ti plasmid pTi15955 of Agrobacterium tumefaciens (database entry X00493) from position 12.541 to 12.350.

Cloning was carried out by isolating the 210 bp SalI/XhoI fragment from pOCS and ligation into the SalI/XhoI-cut vector pJIT117.

This clone is named pJO and was therefore used for cloning into the expression vector pJONP196.

Cloning was effected by isolating the 782 bp SphI fragment from pNP196 and ligation into the SphI-cut vector pJO. The clone which comprises the NP196 ketolase of *Nostoc punctiforme* in the correct orientation as N-terminal translational fusion with the rbcS transit peptide is named pJONP196.

Example 20:
Preparation of expression vectors for the constitutive expression of the NP196-ketolase from *Nostoc punctiforme ATCC 29133* in *Lycopersicon esculentum* and *Tagetes erecta*.

The NP196-ketolase from *Nostoc punctiforme* was expressed in *L. esculentum* and in *Tagetes erecta* under the control of the constitutive promoter FNR (ferredoxin-NADPH oxidoreductase, database entry AB011474, position 70127 to 69493; WO03/006660), from *Arabidopsis thaliana*. The FNR gene starts at base pair 69492

and is annotated as "ferredoxin-NADP+ reductase". The expression was effected with the transit peptide rbcS from pea (Anderson et al. 1986, Biochem J. 240:709-715).

The DNA fragment which comprises the FNR promotor region from *Arabidopsis thaliana* was prepared by means of PCR using genomic DNA (isolated from *Arabidopsis thaliana* by standard methods) and the primers FNR-1 (SEQ ID No. 136) and FNR-2 (SEQ ID No. 137).

The PCR conditions were as follows:

The PCR for the amplification of the DNA which comprises the FNR promoter fragment FNR (SEQ ID No. 138) was carried out in 50 µl of reaction mixture comprising:

- 100 ng genomic DNA from *A.thaliana*
- 0.25 mM dNTPs
- 0.2 mM FNR-1 (SEQ ID No. 136)
- 0.2 mM FNR-2 (SEQ ID No. 137)
- 5 µl 10X PCR buffer (Stratagene)
- 0.25 µl Pfu polymerase (Stratagene)
- 28.8 µl distilled water

The PCR was carried out under the following cycling conditions:

1X	94°C	2 minutes
35X	94°C	1 minute
	50°C	1 minute
	72°C	1 minute
1X	72°C	10 minutes

The 652 bp amplificate was cloned into the PCR cloning vector pCR 2.1 (Invitrogen) using standard methods, giving rise to the plasmid pFNR.

Sequencing of the clone pFNR verified a sequence which agrees with a sequence segment on chromosome 5 of Arabidopsis thaliana (database entry AB011474) from position 70127 to 69493.

This clone is named pFNR and was therefore used for cloning into the expression vector pJONP196 (described in Example 19).

Cloning was effected by isolating the 644 bp SmaI/HindIII fragment from pFNR and ligation into the Ecl136II/HindIII-cut vector pJONP196. The clone which comprises the promoter FNR instead of the original promoter d35S and the fragment NP196 in

the correct orientation as N-terminal fusion with the rbcS transit peptide is named pJOFNR:NP196.

An expression cassette for the Agrobacterium-mediated transformation of the NP196-ketolase from *Nostoc* into *L. esculentum* was generated using the binary vector pSUN3 (WO02/00900).

To generate the expression vector MSP105, the 1839 bp EcoRI/XhoI fragment from pJOFNR:NP196 was ligated with the EcoRI/XhoI-cut vector pSUN3 (Figure 22, construct map). In Figure 22, fragment *FNR promoter* comprises the FNR promoter (635 bp), fragment *rbcS TP FRAGMENT* the rbcS transit peptide from pea (194 bp), fragment *NP196 KETO CDS* (761 bp), encoding the *Nostoc punctiforme* NP196-ketolase, fragment *OCS terminator* (192 bp) the polyadenylation signal of octopine synthase.

An expression cassette for the *Agrobacterium*-mediated transformation of the expression vector with the *Nostoc punctiforme* NP196-ketolase into Tagetes erecta was generated using the binary vector pSUN5 (WO02/00900).

To generate the Tagetes expression vector MSP106, the 1839 bp EcoRI/XhoI fragment from pJOFNR:NP196 was ligated with the EcoRI/XhoI-cut vector pSUN5 (Figure 23, construct map). In Figure 23, fragment *FNR promoter* comprises the FNR promoter (635 bp), fragment *rbcS TP FRAGMENT* the rbcS transit peptide from pea (194 bp), fragment *NP196 KETO CDS* (761 bp), encoding the *Nostoc punctiforme* NP196-ketolase, fragment *OCS terminator* (192 bp) the polyadenylation signal of octopine synthase.

Example 21:
Preparation of expression vectors for the flower-specific expression of the NP196-ketolase from Nostoc *punctiforme ATCC 29133* in *Lycopersicon esculentum* and *Tagetes erecta*

The NP196-ketolase from *Nostoc punctiforme* was expressed in L. esculentum and Tagetes erecta using the transit peptide rbcS from pea (Anderson et al. 1986, Biochem J. 240:709-715). The expression was effected under the control of the flower-specific promoter EPSPS from Petunia hybrida (database entry M37029: nucleotide region 7-1787; Benfey et al. (1990) Plant Cell 2: 849-856).

The DNA fragment which comprises the EPSPS promoter region (SEQ ID No. 141) from Petunia hybrida was prepared by means of PCR using genomic DNA (isolated from Petunia hybrida by standard

methods) and the primers EPSPS-1 (SEQ ID No. 139) and EPSPS-2 (SEQ ID No. 140).

The PCR conditions were as follows:

The PCR for the amplification of the DNA which comprises the EPSPS promoter fragment (database entry M37029: nucleotide region 7-1787) was carried out in 50 µl of reaction mixture comprising:

- 100 ng genomic DNA from *A.thaliana*
- 0.25 mM dNTPs
- 0.2 mM EPSPS-1 (SEQ ID No. 139)
- 0.2 mM EPSPS-2 (SEQ ID No. 140)
- 5 µl 10X PCR buffer (Stratagene)
- 0.25 µl Pfu polymerase (Stratagene)
- 28.8 µl distilled water

The PCR was carried out under the following cycling conditions:

1X	94°C	2 minutes
35X	94°C	1 minute
	50°C	1 minute
	72°C	2 minutes
1X	72°C	10 minutes

The 1773 bp amplificate was cloned into the PCR cloning vector pCR 2.1 (Invitrogen) using standard methods, giving rise to the plasmid pEPSPS.

Sequencing of the clone pEPSPS verified a sequence which differs from the published EPSPS sequence (database entry M37029: nucleotide region 7-1787) only by two deletions (bases ctaagtttcagga in position 46-58 of the sequence M37029; bases aaaaatat in position 1422-1429 of the sequence M37029) and the base substitutions (T instead of G in position 1447 of the sequence M37029; A instead of C in position 1525 of the sequence M37029; A instead of G in position 1627 of the sequence M37029). The two deletions and the two base substitutions at positions 1447 and 1627 of the sequence M37029 were reproduced in an independent amplification experiment and thus represent the actual nucleotide sequence in the Petunia hybrida plants used.

The clone pEPSPS was therefore used for cloning into the expression vector pJONP196 (described in Example 19).

Cloning was effected by isolating the 1763 bp SacI/HindIII fragment from pEPSPS and ligation into the SacI/HindIII-cut vector pJ0NP196. The clone which comprises the promoter EPSPS instead of the original promoter d35S is named pJOESP:NP196. This expression cassette comprises the fragment NP196 in the correct orientation as N-terminal fusion with the rbcS transit peptide.

An expression vector for the Agrobacterium-mediated transformation of the EPSPS-controlled NP196-ketolase from Nostoc punctiforme ATCC 29133 into *L. esculentum* was prepared using the binary vector pSUN3 (WO02/00900).

To prepare the expression vector MSP107, the 2.961 kb SacI/XhoI fragment from pJOESP:NP196 was ligated with the SacI/XhoI-cut vector pSUN3 (Figure 24, construct map). In Figure 24, fragment EPSPS comprises the EPSPS promoter (1761 bp), fragment *rbcS TP FRAGMENT* the rbcS transit peptide from pea (194 bp), fragment *NP196 KETO CDS* (761 bp), encoding the *Nostoc punctiforme* NP196-ketolase, fragment *OCS Terminator* (192 bp) the polyadenylation signal of octopine synthase.

An expression vector for the Agrobacterium-mediated transformation of the EPSPS-controlled NP196-ketolase from Nostoc punctiforme into Tagetes erecta was prepared using the binary vector pSUN5 (WO02/00900).

To prepare the expression vector MSP108, the 2.961 kb SacI/XhoI fragment from pJOESP:NP196 was ligated with the SacI/XhoI-cut vector pSUN5 (Figure 25, construct map). In Figure 25, fragment EPSPS comprises the EPSPS promoter (1761 bp), fragment *rbcS TP FRAGMENT* the rbcS transit peptide from pea (194 bp), fragment *NP196 KETO CDS* (761 bp), encoding the *Nostoc punctiforme* NP196-ketolase, fragment *OCS Terminator* (192 bp) the polyadenylation signal of octopine synthase.

Example 22:
Amplification of a DNA which encodes the entire primary sequence of the NP195-ketolase from *Nostoc punctiforme ATCC 29133*

The DNA which encodes the NP195-ketolase from *Nostoc punctiforme ATCC 29133* was amplified by means of PCR from *Nostoc punctiforme ATCC 29133* (strain of the American Type Culture Collection). The preparation of genomic DNA from a suspension culture of *Nostoc punctiforme ATCC 29133* was described in Example 19.

The nucleic acid encoding a ketolase from *Nostoc punctiforme ATCC 29133* was amplified by means of polymerase chain reaction (PCR) from *Nostoc punctiforme ATCC 29133* using a sense-specific primer (NP195-1, SEQ ID No. 142) and an antisense-specific primer (NP195-2 SEQ ID No. 143).

The PCR conditions were as follows:

The PCR for the amplification of the DNA which encodes a ketolase protein consisting of the entire primary sequence was carried out in 50 µl of reaction mixture comprising:

- 1 µl of a *Nostoc punctiforme ATCC 29133* DNA (prepared as described above)
- 0.25 mM dNTPs
- 0.2 mM NP195-1 (SEQ ID No. 142)
- 0.2 mM NP195-2 (SEQ ID No. 143)
- 5 µl 10X PCR buffer (TAKARA)
- 0.25 µl R Taq polymerase (TAKARA)
- 25.8 µl distilled water

The PCR was carried out under the following cylcing conditions:

1X	94°C	2 minutes
35X	94°C	1 minute
	55°C	1 minute
	72°C	3 minutes
1X	72°C	10 minutes

The PCR amplification with SEQ ID No. 142 and SEQ ID No. 143 resulted in an 819 bp fragment which encodes a protein consisting of the entire primary sequence (NP195, SEQ ID No. 144). The amplificate was cloned into the PCR cloning vector pCR 2.1 (Invitrogen) using standard methods, giving rise to the clone pNP195.

Sequencing of the clone pNP195 with the primers M13F and M13R verified a sequence which is identical to the DNA sequence of 55,604-56,392 of the database entry NZ_AABC010001965, with the exception that T in position 55.604 was replaced by A in order to generate a standard ATG start codon. This nucleotide sequence was reproduced in an independent amplification experiment and thus represents the nucleotide sequence in the *Nostoc punctiforme ATCC 29133* used.

This clone pNP195 was therefore used for cloning into the expression vector pJ0 (described in Example 19). Cloning was effected by isolating the 809 bp SphI fragment from pNP195 and ligation into the SphI-cut vector pJO. The clone which comprises the NP195-ketolase from *Nostoc punctiforme* in the correct orientation as N-terminal translational fusion with the rbcS transit peptide is named pJONP195.

Example 23:

Preparation of expression vectors for the constitutive expression of the NP195-ketolase from *Nostoc punctiforme ATCC 29133* in *Lycopersicon esculentum* and *Tagetes erecta*.

The NP195-ketolase from *Nostoc punctiforme* in *L. esculentum* and in *Tagetes erecta* was expressed under the control of the constitutive promoter FNR (ferredoxin-NADPH oxidoreductase, database entry AB011474 positions 70 127 to 69 493; WO03/006660), from *Arabidopsis thaliana*. The FNR gene starts at base pair 69492 and is annotated as "ferredoxin-NADP+ reductase". The expression was carried out with the transit peptide rbcS from pea (Anderson et al. 1986, Biochem J. 240:709-715).

The clone pFNR (described in Example 20) was therefore used for cloning into the expression vector pJONP195 (described in Example 22).

Cloning was effected by isolating the 644 bp Sma/HindIII fragment from pFNR and ligation into the Ecl136II/HindIII-cut vector pJONP195. The clone which comprises the promoter FNR instead of the original promoter d35S and the fragment NP195 in the correct orientation as N-terminal fusion with the rbcS transit peptide is named pJOFNR:NP195.

An expression cassette for the Agrobacterium-mediated transformation of the NP195-ketolase from *Nostoc punctiforme* in *L. esculentum* was prepared using the binary vector pSUN3 (WO02/00900).

To prepare the expression vector MSP109, the 1866 bp EcoRI/XhoI fragment from pJOFNR:NP195 was ligated with the EcoRI/XhoI-cut vector pSUN3 (Figure 26, construct map). In Figure 26, fragment *FNR promoter* comprises the FNR promoter (635 bp), fragment *rbcS TP FRAGMENT* the rbcS transit peptide from pea (194 bp), fragment *NP195 KETO CDS* (789 bp), encoding the *Nostoc punctiforme* NP195-ketolase, fragment *OCS Terminator* (192 bp) the polyadenylation signal of octopine synthase.

An expression cassette for the *Agrobacterium*-mediated transformation of the expression vector with the NP195-ketolase from Nostoc punctiforme in Tagetes erecta was prepared using the binary vector pSUN5 (WO 02/00900).

To prepare the Tagetes expression vector MSP110, the 1866 bp EcoRI/XhoI fragment from pJOFNR:NP195 was ligated with the EcoRI/XhoI-cut vector pSUN5 (Figure 27, construct map). In Figure 27, fragment *FNR promoter* comprises the FNR promoter (635 bp), fragment rbcS TP FRAGMENT the rbcS transit peptide from pea (194 bp), fragment NP195 KETO CDS (789 bp), encoding the Nostoc punctiforme NP195-ketolase, fragment OCS Terminator (192 bp) the polyadenylation signal of octopine synthase.

Example 24:
Preparation of expression vectors for the flower-specific expression of the NP195-ketolase from Nostoc punctiforme ATCC 29133 in Lycopersicon esculentum and Tagetes erecta.

The NP195-ketolase from *Nostoc punctiforme* was expressed in L. esculentum and Tagetes erecta using the transit peptide rbcS from pea (Anderson et al. 1986, Biochem J. 240:709-715). The expression was effected under the control of the flower-specific promoter EPSPS from Petunia hybrida (database entry M37029: nucleotide region 7-1787; Benfey et al. (1990) Plant Cell 2: 849-856).

The clone pEPSPS (described in Example 21) was therefore used for cloning into the expression vector pJONP195 (described in Example 22).

Cloning was effected by isolating the 1763 bp SacI/HindIII fragment from pEPSPS and ligation into the SacI/HindIII-cut vector pJ0NP195. The clone which comprises the promoter EPSPS instead of the original promoter d35S is named pJOESP:NP195. This expression cassette comprises the fragment NP195 in the correct orientation as N-terminal fusion with the rbcS transit peptide.

An expression vector for the Agrobacterium-mediated transformation of the EPSPS-controlled NP195-ketolase from Nostoc punctiforme ATCC 29133 into *L. esculentum* was prepared using the binary vector pSUN3 (WO02/00900).

To prepare the expression vector MSP111, the 2.988 kb SacI/XhoI fragment from pJOESP:NP196 was ligated with the SacI/XhoI-cut vector pSUN3 (Figure 28, construct map). In Figure 28, fragment EPSPS comprises the EPSPS promoter (1761 bp), fragment *rbcS TP*

FRAGMENT the rbcS transit peptide from pea (194 bp), fragment *NP195 KETO CDS* (789 bp), encoding the *Nostoc punctiforme* NP195-ketolase, fragment *OCS Terminator* (192 bp) the polyadenylation signal of octopine synthase.

An expression vector for the Agrobacterium-mediated transformation of the EPSPS-controlled NP195-ketolase from Nostoc punctiforme into Tagetes erecta was prepared using the binary vector pSUN5 (WO02/00900).

To prepare the expression vector MSP112, the 2.988 kb SacI/XhoI fragment from pJOESP:NP195 was ligated with the SacI/XhoI-cut vector pSUN5 (Figure 29, construct map). In Figure 29, fragment EPSPS comprises the EPSPS promoter (1761 bp), fragment *rbcS TP FRAGMENT* the rbcS transit peptide from pea (194 bp), fragment *NP195 KETO CDS* (789 bp), encoding the *Nostoc punctiforme* NP195-ketolase, fragment *OCS Terminator* (192 bp) the polyadenylation signal of octopine synthase.

Example 25:
Amplification of a DNA which encodes the entire primary sequence of the NODK-ketolase from *Nodularia spumigena NSOR10*.

The DNA which encodes the ketolase from *Nodularia spumigena NSOR10* was amplified by means of PCR from *Nodularia spumigena NSOR10*.

To prepare genomic DNA from a suspension culture of *Nodularia spumigena NSOR10* which had been grown for 1 week under continuous light with constant shaking (150 rpm) at 25°C in *BG 11* medium (1.5 g/l $NaNO_3$, 0.04 g/l $K_2PO_4x3H_2O$, 0.075 g/l $MgSO_4xH_2O$, 0.036 g/l $CaCl_2x2H_2O$, 0.006 g/l citric acid, 0.006 g/l ferric ammonium citrate, 0.001 g/l EDTA disodium magnesium, 0.04 g/l Na_2CO_3, 1 ml Trace Metal Mix "A5+Co" (2.86 g/l H_3BO_3, 1.81 g/l $MnCl_2x4H_2O$, 0.222 g/l $ZnSO_4x7H_2O$, 0.39 g/l $NaMoO_4X2H_2O$, 0.079 g/l $CuSO_4x5H_2O$, 0.0494 g/l $Co(NO_3)_2x6H_2O$), the cells were harvested by centrifugation, frozen in liquid nitrogen and ground to a powder in a mortar.

Protocol for the DNA isolation from *Nodularia spumigena NSOR10*:

The bacterial cells were pelleted from a 10 ml liquid culture by centrifugation for 10 minutes at 8000 rpm. Thereafter, the bacterial cells were crushed and ground in liquid nitrogen using a mortar. The cell material was resuspended in 1 ml of 10 mM Tris-HCl (pH 7.5) and transferred to an Eppendorf reaction vessel (volume 2 ml). After addition of 100 µl of Proteinase K

(concentration: 20 mg/ml), the cell suspension was incubated for 3 hours at 37°C. Thereafter, the suspension was extracted with 500 µl of phenol. After centrifugation for 5 minutes at 13 000 rpm, the aqueous top phase was transferred to a fresh 2 ml Eppendorf reaction vessel. The phenol extraction was repeated 3 times. The DNA was precipitated by addition of 1/10 volume 3 M sodium acetate (pH 5.2) and 0.6 volume isopropanol and subsequently washed with 70% ethanol. The DNA pellet was dried at room temperature, taken up in 25 µl of water and dissolved with heating at 65°C.

The nucleic acid encoding a ketolase from *Nodularia spumigena NSOR10* was amplified from *Nodularia spumigena NSOR10* by means of polymerase chain reaction (PCR) using a sense-specific primer (NODK-1, SEQ ID No. 146) and an antisense-specific primer (NODK-2, SEQ ID No. 147).

The PCR conditions were as follows:

The PCR for the amplification of the DNA which encodes a ketolase protein consisting of the entire primary sequence was carried out in 50 µl of reaction mixture comprising:

- 1 µl of a *Nodularia spumigena NSOR10* DNA (prepared as described above)
- 0.25 mM dNTPs
- 0.2 mM NODK-1 (SEQ ID No. 146)
- 0.2 mM NODK-2 (SEQ ID No. 147)
- 5 µl 10X PCR buffer (TAKARA)
- 0.25 µl R Taq polymerase (TAKARA)
- 25.8 µl distilled water

The PCR was carried out under the following cycling conditions:

1X	94°C	2 minutes
35X	94°C	1 minute
	55°C	1 minute
	72°C	3 minutes
1X	72°C	10 minutes

The PCR amplification with SEQ ID No. 146 and SEQ ID No. 147 resulted in a 720 bp fragment which encodes a protein consisting of the entire primary sequence (NODK, SEQ ID No. 148). The amplificate was cloned into the PCR cloning vector pCR 2.1 (Invitrogen) using standard methods, giving rise to the clone pNODK.

Sequencing of the clone pNODK with the M13F and the M13R primer verified a sequence which is identical to the DNA sequence of 2130-2819 of the database entry AY210783 (with inverse orientation relative to the published database entry). This nucleotide sequence was reproduced in an independent amplification experiment and thus represents the nucleotide sequence in the *Nodularia spumigena NSOR10* used.

This clone pNODK was therefore used for cloning into the expression vector pJ0 (described in Example 19). Cloning was effected by isolating the 710 bp SphI fragment from pNODK and ligation into the SphI-cut vector pJO. The clone which comprises the NODK-ketolase from *Nodularia spumigena* in the correct orientation as N-terminal translational fusion with the rbcS transit peptide is named pJONODK.

Example 26:
Preparation of expression vectors for the constitutive expression of the NODK-ketolase from *Nodularia spumigena NSOR10* in *Lycopersicon esculentum* and *Tagetes erecta*.

The NODK-ketolase from *Nodularia spumigena NSOR10* was expressed in *L. esculentum* and in *Tagetes erecta* under the control of the constitutive promoter FNR (ferredoxin-NADPH oxidoreductase, database entry AB011474, position 70127 to 69493; WO03/006660), from *Arabidopsis thaliana*. The FNR gene starts at base pair 69 492 and is annotated as "ferredoxin-NADP+ reductase". The expression was effected with the transit peptide rbcS from pea (Anderson et al. 1986, Biochem J. 240:709-715).

The clone pFNR (described in Example 20) was therefore used for cloning into the expression vector pJONODK (described in Example 25).

Cloning was effected by isolating the 644 bp SmaI/HindIII fragment from pFNR and ligation into the Ecl136II/HindIII-cut vector pJONODK. The clone which comprises the promoter FNR instead of the original promoter d35S and the fragment NODK in the correct orientation as N-terminal fusion with the rbcS transit peptide is named pJOFNR:NODK.

An expression cassette for the Agrobacterium-mediated transformation of the NODK-ketolase from *Nodularia spumigena NSOR10* into *L. esculentum* was generated using the binary vector pSUN3 (WO02/00900).

To generate the expression vector MSP113, the 1 767 bp EcoRI/XhoI fragment from pJOFNR:NODK was ligated with the EcoRI/XhoI-cut vector pSUN3 (Figure 30, construct map). In Figure 30, fragment *FNR promoter* comprises the FNR promoter (635 bp), fragment *rbcS TP FRAGMENT* the rbcS transit peptide from pea (194 bp), fragment *NODK KETO CDS* (690 bp), encoding the *Nodularia spumigena NSOR10* NODK-ketolase, fragment *OCS terminator* (192 bp) the polyadenylation signal of the octopine synthase.

An expression cassette for the *Agrobacterium*-mediated transformation of the expression vector with the *Nodularia spumigena NSOR10* punctiforme NODK-ketolase into Tagetes erecta was generated using the binary vector pSUN5 (WO02/00900).

To generate the Tagetes expression vector MSP114, the 1767 bp EcoRI/XhoI fragment from pJOFNR:NODK was ligated with the EcoRI/XhoI-cut vector pSUN5 (Figure 31, construct map). In Figure 31, fragment *FNR promoter* comprises the FNR promoter (635 bp), fragment rbcS TP FRAGMENT the rbcS transit peptide from pea (194 bp), fragment NODK KETO CDS (690 bp), encoding the *Nodularia spumigena NSOR10* NODK-ketolase, fragment OCS terminator (192 bp) the polyadenylation signal of octopine synthase.

Example 27:

Preparation of expression vectors for the flower-specific expression of the NODK-ketolase from *Nodularia spumigena NSOR10* in *Lycopersicon esculentum* and *Tagetes erecta*

The NODK-ketolase from *Nodularia spumigena NSOR10* was expressed in L. esculentum and Tagetes erecta using the transit peptide rbcS from pea (Anderson et al. 1986, Biochem J. 240:709-715). The expression was effected under the control of flower-specific promoter EPSPS from Petunia hybrida (database entry M37029: nucleotide region 7-1787; Benfey et al. (1990) Plant Cell 2: 849-856).

Clone pEPSPS (described in Example 21) was therefore used for cloning into the expression vector pJONODK (described in Example 25).

Cloning was effected by isolating the 1763 bp SacI/HindIII fragment from pEPSPS and ligation into the SacI/HindIII-cut vector pJ0NODK. The clone which comprises the promoter EPSPS instead of the original promoter d35S is named pJOESP:NODK. This expression cassette comprises the fragment NODK in the correct orientation as N-terminal fusion with the rbcS transit peptide.

An expression vector for the Agrobacterium-mediated transformation of the EPSPS-controlled NODK-ketolase from *Nodularia spumigena NSOR10* into *L. esculentum* was prepared using the binary vector pSUN3 (WO02/00900).

To prepare the expression vector MSP115, the 2.889 kb SacI/XhoI fragment from pJOESP:NODK was ligated with the SacI/XhoI-cut vector pSUN3 (Figure 32, construct map). In Figure 32, fragment EPSPS comprises the EPSPS promoter (1761 bp), fragment *rbcS TP FRAGMENT* the rbcS transit peptide from pea (194 bp), fragment *NODK KETO CDS* (690 bp), encoding the *Nodularia spumigena NSOR10* NODK-ketolase, fragment *OCS Terminator* (192 bp) the polyadenylation signal of octopine synthase.

An expression vector for the Agrobacterium-mediated transformation of the EPSPS-controlled NODK-ketolase from *Nodularia spumigena NSOR10* into Tagetes erecta was prepared using the binary vector pSUN5 (WO02/00900).

To prepare the expression vector MSP116, the 2.889 kb SacI/XhoI fragment from pJOESP:NODK was ligated with the SacI/XhoI-cut vector pSUN5 (Figure 33, construct map). In Figure 33, fragment EPSPS comprises the EPSPS promoter (1761 bp), fragment *rbcS TP FRAGMENT* the rbcS transit peptide from pea (194 bp), fragment *NODK KETO CDS* (690 bp), encoding the *Nodularia spumigena NSOR10* NODK-ketolase, fragment *OCS Terminator* (192 bp) the polyadenylation signal of octopine synthase.

Example 28:
Generation of transgenic Lycopersicon esculentum plants

Tomato plants were transformed and regenerated as described in Example 6.

In accordance with the transformation method described in Example 6, the following lines were obtained with the following expression constructs:

the following were obtained with MSP105: msp105-1, msp105-2, msp105-3

the following were obtained with MSP107: msp107-1, msp107-2, msp107-3

the following were obtained with MSP109: msp109-1, msp109-2, msp109-3

the following were obtained with MSP111: msp111-1, msp111-2, msp111-3

the following were obtained with MSP113: msp113-1, msp113-2, msp113-3
the following were obtained with MSP115: msp115-1, msp115-2, msp115-3

The characterization and analysis of the transgenic Lycopersicon esculentum plants is carried out as described in Example 6.

Example 29:
Generation of transgenic Tagetes plants

Tagetes plants were transformed and regenerated as described in Example 7.

In accordance with the transformation method described in Example 7, the following lines were obtained with the following expression constructs:

the following were obtained with MSP106: msp106-1, msp106-2, msp106-3

the following were obtained with MSP108: msp108-1, msp108-2, msp108-3
the following were obtained with MSP110: msp110-1, msp110-2, msp110-3
the following were obtained with MSP112: msp112-1, msp112-2, msp112-3

the following were obtained with MSP114: msp114-1, msp114-2, msp114-3
the following were obtained with MSP116: msp116-1, msp116-2, msp116-3

The transgenic Tagetes plants were characterized as described in Examples 8 and 9 and in Example 17.

Example 30:
Preparation of a double expression vector for downregulating the epsilon-cyclase transcript quantities and for expressing the *Nostoc punctiforme* ketolase NP196-1 in *Tagetes erecta* in a flower-specific manner.

Cloning was carried out by isolating the 2963 bp Ecl136II/XhoI fragment from MSP107 (see Example 21) and ligation with the XhoI/SmaI-cut vector pS5AI7 (Example 14). The ligation gives rise to a T-DNA which comprises two expression cassettes: firstly, the inverted-repeat cassette which is directed against the epsilon-cyclase from Tagetes erecta and, secondly, a cassette for overexpressing the ketolase NP196-1 from *Nostoc punctiforme*. This clone is named pCSP01 (Figure 34, construct map). In Figure 34, fragment *AP3P* (776 bp) comprises the AP3P promoter, fragment *ecycS* (439 bp) the 5'region of the Tagetes epsilon-cyclase sequence from pJIT117, fragment *intron* (207 bp) the intron PIV2 of the potato gene ST-LS1, fragment *ecycAS* (440 bp) the 5'region of the epsilon-cyclase from Tagetes erecta in antisense orientation, fragment *35T* (763 bp) the polyadenylation signal of CaMV. Furthermore, fragment *ocs* (191 bp) comprises the polyadenylation signal of the octopine synthase gene, fragment *NP196* (762 bp) the ketolase from *Nostoc punctiforme*, fragment *TP* (183 bp) the transit peptide of the rbcS gene from pea, and fragment *EPSPS* (1761 bp) the EPSPS promoter.

Example 31:
Preparation of an expression cassette for the flower-specific overexpression of the chromoplast-specific β-hydroxylase from *Lycopersicon esculentum*.

The chromoplast-specific β-hydroxylase from *Lycopersicon esculentum* is expressed in *Tagetes erecta* under the control of the flower-specific promoter EPSPS from petunia (Example 21). The terminator element used is LB3 from Vicia faba. The sequence of the chromoplast-specific β-hydroxylase was prepared by isolating RNA, reverse transcription and PCR.

To prepare the LB3 terminator sequence from *Vicia faba*, genomic DNA is isolated from *Vicia faba* tissue following standard methods and employed by genomic PCR using the primers PR206 and PR207. The PCR for the amplification of this LB3 DNA fragment is carried out in 50 µl of reaction mixture comprising:

- 1 µl cDNA (prepared as described above)
- 0.25 mM dNTPs
- 0.2 µM PR206 (SEQ ID No. 150)
- 0.2 µM PR207 (SEQ ID No. 151)
- 5 µl 10X PCR buffer (TAKARA)
- 0.25 µl R Taq polymerase (TAKARA)
- 28.8 µl distilled water

The PCR amplification with PR206 and PR207 results in a 0.3 kb fragment which comprises the LB terminator. The amplificate is cloned into the cloning vector pCR-BluntII (Invitrogen). Sequencing reactions with the primers T7 and M13 confirm a sequence which is identical to the sequence SEQ ID NO.: 160. This clone is named pTA-LB3 and is therefore used for cloning into the vector pJIT117 (see herein below).

To prepare the β-hydroxylase sequence, total RNA is prepared from tomato. To this end, 100 mg of the frozen, pulverized flowers are transferred into a reaction vessel and taken up in 0.8 ml of Trizol buffer (LifeTechnologies). The suspension is extracted with 0.2 ml of chloroform. After centrifugation for 15 minutes at 12 000 g, the aqueous supernatant is removed, transferred into a fresh reaction vessel and extracted with one volume of ethanol. The RNA is precipitated with one volume of isopropanol, washed with 75% of ethanol, and the pellet is dissolved in DEPC water (overnight incubation of water with 1/1000 volume of diethyl pyrocarbonate at room temperature, followed by autoclaving). The RNA concentration is determined photometrically. For the cDNA synthesis, 2.5 µg of total RNA are denatured for 10 minutes at 60°C, cooled on ice for 2 minutes and transcribed into cDNA by means of a cDNA kit (Ready-to-go-you-prime-beads, Pharmacia Biotech) following the manufacturer's instructions using an antisense-specific primer (PR215 SEQ ID No. 152).

The conditions of the subsequent PCR reactions are as follows:

The PCR for the amplification of the VPR203-PR215 DNA fragment, which encodes the β-hydroxylase, is carried out in 50 µl of reaction mixture comprising:

- 1 µl cDNA (prepared as described above)
- 0.25 mM dNTPs
- 0.2 µM VPR203 (SEQ ID No. 159)
- 0.2 µM PR215 (SEQ ID No. 152)
- 5 µl 10X PCR buffer (TAKARA)
- 0.25 µl R Taq polymerase (TAKARA)
- 28.8 µl distilled water

The PCR amplification with VPR203 and PR215 results in a 0.9 kb fragment which encodes the β-hydroxylase. The amplificate is cloned into the cloning vector pCR-BluntII (Invitrogen). Sequencing reactions with the primers T7 and M13 confirm a sequence which is identical to the sequence SEQ ID NO.: 161. This

clone is named pTA-CrtR-b2 and is therefore used for cloning into the vector pCSP02 (see herein below).

The EPSPS promoter sequence from petunia is prepared by PCR amplification using the plasmid MSP107 (see Example 21) and the primers VPR001 and VPR002. The PCR for the amplification of this EPSPS DNA fragment is carried out in 50 µl of reaction mixture comprising:

- 1 µl cDNA (prepared as described above)
- 0.25 mM dNTPs
- 0.2 µM VPR001 (SEQ ID No. 157)
- 0.2 µM VPR002 (SEQ ID No. 158)
- 5 µl 10X PCR buffer (TAKARA)
- 0.25 µl R Taq polymerase (TAKARA)
- 28.8 µl distilled water

The PCR amplification with VPR001 and PR002 results in a 1.8 kb fragment which encodes the EPSPS promoter. The amplificate is cloned into the cloning vector pCR-BluntII (Invitrogen). Sequencing reactions with the primers T7 and M13 confirm a sequence which is identical to the sequence SEQ ID NO.: 162. This clone is named pTA-EPSPS and is therefore used for cloning into the vector pCSP03 (see herein below).

The first cloning step is effected by isolating the 0.3 kb PR206-PR207 EcoRI/XhoI fragment from pTA-LB3, derived from the cloning vector pCR-BluntII (Invitrogen), and ligation with the EcoRI/XhoI-cut vector pJIT117. The clone which comprises the 0.3 kb terminator LB3 is named pCSP02.

The second cloning step is effected by isolating the 0.9 kb VPR003-PR215 EcoRI/HindIII fragment from pTA-CrtR-b2, derived from the cloning vector pCR-BluntII (Invitrogen), and ligation with the EcoRI/HindIII-cut vector pcsp02. The clone which comprises the 0.9 kb β-hydroxylase fragment CrtR-b2 is named pCSP03. The ligation gives rise to a transcriptional fusion between the terminator LB3 and the β-hydroxylase fragment CrtR-b2.

The third cloning step is effected by isolating the 1.8 kb VPR001-VPR002 NcoI/SacI fragment from pTA-EPSPS, derived from the cloning vector pCR-BluntII (Invitrogen), and ligation with the NcoI/SacI-cut vector pCSP03. The clone which comprises the 1.8 kb EPSPS promoter fragment is named pCSP04. The ligation gives rise to a transcriptional fusion between the EPSPS promoter and the β-hydroxylase fragment CrtR-b2, (Figure 35, construct map). In Figure 35, fragment *EPSPS* (1792 bp) comprises the EPSPS promoter,

fragment *crtRb2* (929 bp) the β-hydroxylase CrtRb2, fragment *LB3* (301 bp) the LB3 terminator.

To clone this β-hydroxylase overexpression cassette into expression vectors for the Agrobacterium-mediated transformation of *Tagetes erecta*, the β-hydroxylase cassette is isolated as a 3103 bp Ecl136II/XhoI fragment. The 3' ends are filled in (30 minutes at 30°C) by standard methods (Klenow fill-in).

Example 32:
Preparation of inverted-repeat expression cassettes for the flower-specific expression of β-hydroxylase dsRNA in *Tagetes erecta* (directed against the 5' region of the β-hydroxylase cDNA)

The nucleic acid which comprises the 5'-terminal bp region of the β-hydroxylase cDNA (Genbank accession no. AF251018) is amplified from *Tagetes erecta* cDNA by means of polymerase chain reaction (PCR) using a sense-specific primer (PR217 SEQ ID No. 153) and an antisense-specific primer (PR218 SEQ ID No. 154).

To prepare total RNA of Tagetes flowers, 100 mg of the frozen, pulverized flowers are transferred into a reaction vessel and taken up in 0.8 ml of Trizol buffer (LifeTechnologies). The suspension is extracted with 0.2 ml of chloroform. After centrifugation for 15 minutes at 12 000 g, the aqueous supernatant is removed, transferred into a fresh reaction vessel and extracted with one volume of ethanol. The RNA is precipitated with one volume of isopropanol, washed with 75% of ethanol, and the pellet is dissolved in DEPC water (overnight incubation of water with 1/1000 volume of diethyl pyrocarbonate at room temperature, followed by autoclaving). The RNA concentration is determined photometrically. For the cDNA synthesis, 2.5 µg of total RNA are denatured for 10 minutes at 60°C, cooled on ice for 2 minutes and transcribed into cDNA by means of a cDNA kit (Ready-to-go-you-prime-beads, Pharmacia Biotech) following the manufacturer's instructions using an antisense-specific primer (PR218 SEQ ID No. 154).

The conditions of the subsequent PCR reactions are as follows:

The PCR for the amplification of the PR217-PR218 DNA fragment, which encodes the 5'-terminal 0.3 kb region of the β-hydroxylase, is carried out in 50 µl of reaction mixture comprising:

- 1 µl cDNA (prepared as described above)
- 0.25 mM dNTPs
- 0.2 µM PR217 (SEQ ID No. 153)

- 0.2 µM PR218 (SEQ ID No. 154)
- 5 µl 10X PCR buffer (TAKARA)
- 0.25 µl R Taq polymerase (TAKARA)
- 28.8 µl distilled water

The PCR for the amplification of the PR220-PR219 DNA fragment, which encodes the 5'-terminal 0.3 kb region of the β-hydroxylase, is carried out in 50 µl of reaction mixture comprising:

- 1 µl cDNA (prepared as described above)
- 0.25 mM dNTPs
- 0.2 µM PR220 (SEQ ID No. 156)
- 0.2 µM PR219 (SEQ ID No. 155)
- 5 µl 10X PCR buffer (TAKARA)
- 0.25 µl R Taq polymerase (TAKARA)
- 28.8 µl distilled water

The PCR reactions are carried out under the following cycling conditions:

1X	94°C	2 minutes
35X	94°C	1 minute
	58°C	1 minute
	72°C	1 minute
1X	72°C	10 minutes

The PCR amplification with the primers PR217 and PR218 results in a 332 bp fragment (SEQ ID NO: 163), and the PCR amplification with the primers PR219 and PR220 results in a 332 bp fragment (SEQ ID NO: 164).

The two amplificates, viz. the PR217-PR218 (HindIII/SalI sense) fragment and the PR220-PR219 (EcoRI/BamHI antisense) fragment, are cloned into the PCR cloning vector pCR-BluntII (Invitrogen) using standard conditions. The resulting clones are named pCR-BluntII-bhydrS (PR217-PR218 fragment) and pCR-BluntII-bhydrAS (PR220-PR219 fragment). Sequencing reactions with the primer SP6 confirm in each case a sequence which is identical to the published sequence AF251018 (SEQ ID No. 165), with the exception of the restriction sites which had been introduced. These clones are therefore used for the preparation of an inverted-repeat construct in the cloning vector pJAI1 (see Example 10).

The first cloning step is effected by isolating the 332 bp PR217-PR218 HindIII/SalI fragment from the cloning vector pCR-BluntII-bhydrS (Invitrogen) and ligation with the HindIII/SalI-cut vector pJAI1. The clone which comprises the

5'-terminal region of the β-hydroxylase in sense orientation is named pCSP05. The ligation gives rise to a transcriptional fusion between the AP3P and the sense fragment of the 5'-terminal region of the β-hydroxylase and, secondly, the intron.

The second cloning step is effected by isolating the 332 bp PR220-PR219 BamHI-EcoRI fragment from the cloning vector pCR-BluntII-bhydrAS (Invitrogen) and ligation with the BamHI-EcoRI-cut vector pCSP05. The clone which comprises the 332 bp 5'-terminal region of the β-hydroxylase cDNA in antisense orientation is named pCSP06. The ligation gives rise to a transcriptional fusion between, firstly, the antisense fragment of the 5'-terminal region of the β-hydroxylase and the polyadenylation signal from CaMV and, secondly, the intron.

To clone this downregulating cassette into expression vectors for the Agrobacterium-mediated transformation of *Tagetes erecta*, the inverted-repeat cassette is isolated as a 2394 bp Ecl136II/XhoI fragment. The 3' ends are filled in (30 minutes at 30°C) by standard methods (Klenow fill-in).

In Figure 36, fragment *AP3P* (767 bp) comprises the AP3P promoter, fragment *5'bhydrS* (291 bp) the 5' region of the β-hydroxylase from *Tagetes erecta* in sense orientation, fragment *intron* (206 bp) the intron PIV2 of the potato gene ST-LS1, fragment *5'bhydrS* (326 bp) the 5' region of the β-hydroxylase from *Tagetes erecta* in antisense orientation, and fragment *35T* (761 Bp) the polyadenylation signal of CaMV.

To clone this downregulating cassette into expression vectors for the Agrobacterium-mediated transformation of *Tagetes erecta*, the inverted-repeat cassette is isolated as a 2392 bp Ecl136II/XhoI fragment. The 3' ends are filled in (30 minutes at 30°C) by standard methods (Klenow fill-in).

Example 33:
Preparation of a triple expression vector for downregulating the epsilon-cyclase, for expressing the *Nostoc punctiforme* ketolase NP196-1 and for overexpressing the chromoplast-specific β-hydroxylase from *Lycopersicon esculentum* in a flower-specific manner in *Tagetes erecta*.

Cloning of this triple expression vector is effected by isolating the 3103 bp Ecl136II/XhoI fragment from pCSP04 (see Example 31), subsequent Klenow fill-in the of 5' overhang of the XhoI cleavage site (carried out by standard methods) and, finally, ligation in the Ecl136II-cut vector pCSP01 (Example 30). The ligation gives

rise to a T-DNA which comprises three expression cassettes: firstly, the inverted-repeat cassette directed against the epsilon-cyclase from Tagetes erecta, secondly a cassette for overexpressing the ketolase NP196-1 from *Nostoc punctiforme*, and, thirdly, a cassette for the chromoplast-specific overexpression of the β-hydroxylase from *Lycopersicon esculentum*. The β-hydroxylase overexpression cassette can ligate into the vector in two orientations. The example pCSP07, which is described herein, comprises both resulting versions of the triple expression vector, pCSP07F and pCSP07R.

By way of representation, the construct map for version pCSP07F of the example pCSP07 is shown herein (Figure 37, construct map).

In Figure 37, fragment *AP3P* (773 bp) comprises the AP3P promoter, fragment *ecycS* (439 bp) the 5' region of the epsilon-cyclase sequence from Tagetes erecta in sense orientation, fragment *intron* (207 bp) the intron PIV2 of the potato gene ST-LS1, fragment *ecycAS* (440 bp) the 5' region of the epsilon-cyclase from Tagetes erecta in antisense orientation, and fragment *35T* (763 bp) the polyadenylation signal of CaMV.

Furthermore, the fragment *ocs* (191 bp) comprises the polyadenylation signal of the octopine synthase gene, fragment *NP196* (762 bp) the ketolase from *Nostoc punctiforme*, fragment *TP* (183 bp) the transit peptide of the rbcS gene from pea, fragment *EPSPS* (1761 bp) the EPSPS promoter.

Furthermore, fragment *EPSPS* (1792 bp) comprises the EPSPS promoter, fragment *crtRb2* (929 bp) the β-hydroxylase CrtRb2, fragment *LB3* (301 bp) the LB3 terminator.

Transformation and regeneration of Tagetes plants were described in Example 7.

Example 34:
Preparation of a quadruple expression vector for downregulating the epsilon-cyclase, for expressing the *Nostoc punctiforme* ketolase NP196-1, for overexpressing chromoplast-specific β-hydroxylase from *Lycopersicon esculentum* and for downregulating the β-hydroxylase from *Tagetes erecta* in *Tagetes erecta* in a flower-specific manner.

Cloning of this quadruple expression vector is effected by isolating the 2392 bp Ecl136II/XhoI fragment from pCSP06 (see Example 32), subsequent Klenow fill-in of the 5' overhang of the

XhoI cleavage site (carried out by standard methods) and, finally, ligation in the Ecl136II-cut vector pCSP07 (Example 33). The ligation gives rise to a T-DNA which comprises four expression cassettes: firstly, the inverted-repeat cassette directed against the epsilon-cyclase from Tagetes erecta, secondly a cassette for overexpressing the ketolase NP196-1 from *Nostoc punctiforme*, thirdly a cassette for the chromoplast-specific overexpression of the β-hydroxylase from *Lycopersicon esculentum* and, fourthly, an inverted-repeat cassette directed against the β-hydroxylase from Tagetes erecta. The β-hydroxylase downregulation cassette can ligate into the vector in two orientations. The example pCSP08, which is described herein, comprises both resulting versions of the quadruple expression vector, pCSP08F and pCSP08R.

By way of representation, the construct map for version pCSP08F of the example pCSP08 is shown herein (Figure 38, construct map). In Figure 38, fragment *AP3P* (773 bp) comprises the AP3P promoter, fragment *ecycS* (439 bp) the 5' region of the epsilon-cyclase sequence from Tagetes erecta in sense orientation, fragment *intron* (207 bp) the intron PIV2 of the potato gene ST-LS1, fragment *ecycAS* (440 bp) the 5' region of the epsilon-cyclase from Tagetes erecta in antisense orientation, fragment *35T* (763 bp) the polyadenylation signal of CaMV.

Furthermore, fragment *ocs* (191 bp) comprises the polyadenylation signal of the octopine synthase gene, fragment *NP196* (762 bp) the ketolase from *Nostoc punctiforme*, fragment *TP* (183 bp) the transit peptide of the rbcS gene from pea, and fragment *EPSPS* (1761 bp) the EPSPS promoter.

Furthermore, fragment *EPSPS* (1792 bp) comprises the EPSPS promoter, fragment *crtRb2* (929 bp) the β-hydroxylase CrtRb2, fragment *LB3* (301 bp) the LB3 terminator.

Furthermore, fragment *AP3P* (767 bp) comprises the AP3P promoter, fragment *5'bhydrS* (291 bp) the 5' region of the β-hydroxylase from *Tagetes erecta* in sense orientation, fragment *intron* (206 bp) the intron PIV2 of the potato gene ST-LS1, fragment *5'bhydrS* (326 bp) the 5' region of the β-hydroxylase from *Tagetes erecta* in antisense orientation, and fragment *35T* (761 bp) the polyadenylation signal from CaMV.

Example 35:

Preparation of a quintuple expression vector for downregulating the epsilon-cyclase, for expressing the *Nostoc punctiforme* ketolase NP196-1, for overexpressing the chromoplast-specific

β-hydroxylase from *Lycopersicon esculentum*, for downregulating the β-hydroxylase from *Tagetes erecta* and for overexpressing the Bgenes from tomato in *Tagetes erecta* in a flower-specific manner.

Cloning of this quintuple expression vector is effected by isolating the 2679 bp PmeI/SspI fragment from pMKP1 (see Example 37), and ligation in the Ecl136II-cut vector pCSP08 (Example 34). The ligation gives rise to a T-DNA which comprises five expression cassettes: firstly, the inverted-repeat cassette directed against the epsilon-cyclase from Tagetes erecta, secondly a cassette for overexpressing the ketolase NP196-1 from *Nostoc punctiforme*, thirdly a cassette for the chromoplast-specific overexpression of the β-hydroxylase from *Lycopersicon esculentum*, fourthly an inverted-repeat cassette directed against the β-hydroxylase from Tagetes erecta and, fifthly, a cassette for overexpressing the Bgene from *Lycopersicon esculentum*. The β-hydroxylase downregulation cassette can ligate into the vector pCSP08 in two orientations. The example pCSP09, which is described herein, comprises both resulting versions of the quadruple expression vector, pCSP09F and pCSP09R.

By way of representation, the construct map for version pCSP09F of the example pCSP09 is shown herein (Figure 39, construct map). In Figure 39, fragment *AP3P* (773 bp) comprises the AP3P promoter, fragment *ecycS* (439 bp) the 5' region of the epsilon-cyclase sequence from Tagetes erecta in sense orientation, fragment *intron* (207 bp) the intron PIV2 of the potato gene ST-LS1, fragment *ecycAS* (440 bp) the 5' region of the epsilon-cyclase from Tagetes erecta in antisense orientation, fragment *35T* (763 bp) the polyadenylation signal of CaMV.

Furthermore, fragment *ocs* (191 bp) comprises the polyadenylation signal of the octopine synthase gene, fragment *NP196* (762 bp) the ketolase from *Nostoc punctiforme*, fragment *TP* (183 bp) the transit peptide of the rbcS gene from pea, and fragment *EPSPS* (1761 bp) the EPSPS promoter.

Furthermore, fragment *EPSPS* (1792 bp) comprises the EPSPS promoter, fragment *crtRb2* (929 bp) the β-hydroxylase CrtRb2, fragment *LB3* (301 bp) the LB3 terminator.

Furthermore, fragment *AP3P* (767 bp) comprises the AP3P promoter, fragment *5'bhydrS* (291 bp) the 5' region of the β-hydroxylase from *Tagetes erecta* in sense orientation, fragment *intron* (206 bp) the intron PIV2 of the potato gene ST-LS1, fragment *5'bhydrS* (326 bp) the 5' region of the β-hydroxylase from *Tagetes erecta* in

antisense orientation, and fragment *35T* (761 bp) the polyadenylation signal from CaMV.

Furthermore, fragment P76 (1033 bp) comprises the P76 promoter, fragment Bgene (1666 bp) the Bgene from *Lycopersicon esculentum*, and fragment 35ST (970 bp) the polyadenylation signal from CaMV.

Example 36:

Preparation of a quadruple expression vector for expressing the *Nostoc punctiforme* ketolase NP196-1, for overexpressing the chromoplast-specific β-hydroxylase from *Lycopersicon esculentum*, for downregulating the β-hydroxylase from *Tagetes erecta* and for overexpressing the Bgenes from tomato in *Tagetes erecta* in a flower-specific manner.

The first cloning step is effected by isolating the 3103 bp Ecl136II/XhoI fragment from pCSP04, followed by Klenow fill-in of the 5' overhang of the XhoI cleavage site (carried out by standard methods) and, finally, ligation into the Ecl136II-cut vector pMSP107. The ligation gives rise to a T-DNA which comprises two expression cassettes: firstly, the cassette for overexpressing the ketolase NP196-1 from *Nostoc punctiforme*, and, secondly, the cassette for overexpressing the chromoplast-specific β-hydroxylase from *Lycopersicon esculentum*. The β-hydroxylase overexpression cassette can ligate into the vector pMSP107 in two orientations. The example pCSP010 which is described herein comprises both resulting versions of the double expression vector, pCSP10F and pCSP10R.

The second cloning step is effected by isolating the 2392 bp Ecl136II/XhoI fragmente from pCSP06, followed by Klenow fill-in of the 5' overhang of the XhoI cleavage site (carried out by standard methods), and, finally, ligation into the Ecl136II-cut vector pCSP10. The β-hydroxylase downregulation cassette can ligate into the vector pCSP10 in two orientations. The example pCSP11 which is described herein comprises both resulting versions of the triple expression vector pCSP11F and pCSP11R.

The third cloning step is effected by isolating the 3679 bp PmeI/SspI fragment from pMKP01 (see Example 37) and ligation into the Ecl136II-cut vector pCSP11. The Bgene overexpression cassette can ligate into the vector pCSP11 in two orientations. The example pCSP12 which is described herein comprises both resulting versions of the quadruple expression vector, pCSP12F and pCSP12R.

By way of representation, the construct map for version pCSP12F of the example pCSP12 is shown herein (Figure 40, construct map).

In Figure 40, fragment *ocs* (191 bp) comprises the polyadenylation signal of the octopine synthase gene, fragment *NP196* (762 bp) the ketolase from *Nostoc punctiforme*, fragment *TP* (183 bp) the transit peptide of the rbcS gene from pea, and fragment *EPSPS* (1761 bp) the EPSPS promoter.

Furthermore, fragment *EPSPS* (1792 bp) comprises the EPSPS promoter, fragment *crtR-b2* (929 bp) the β-hydroxylase CrtRb2, and fragment *LB3* (301 bp) the LB3 terminator.

Furthermore, fragment *AP3P* (767 bp) comprises the AP3P promoter, fragment *5'bhydrS* (291 bp) the 5' region of the β-hydroxylase from *Tagetes erecta* in sense orientation, fragment *intron* (206 bp) the intron PIV2 of the potato gene ST-LS1, fragment *5'bhydrS* (326 bp) the 5' region of the β-hydroxylase from *Tagetes erecta* in antisense orientation, and fragment *35T* (761 bp) the polyadenylation signal from CaMV.

Furthermore, fragment P76 (1033 bp) comprises the P76 promoter, the fragment *Bgene* (1666 bp) the Bgene from *Lycopersicon esculentum*, and the fragment *35ST* (970 bp) the polyadenylation signal from CaMV.

Example 37:
Preparation of expression vectors for the flower-specific expression of the chromoplast-specific lycopene beta-cyclase from Lycopersicon esculentum under the control of the promoter P76 and for the flower-specific expression of the ketolase NP196 from *Nostoc punctiforme ATCC 29133* under the control of the EPSPS promoter

Isolation of promoter P76 (SEQ ID NO. 168) by means of PCR with genomic DNA from Arabidopsis thaliana as template.

The oligonucleotide primers P76for (SEQ ID NO. 166) and P76rev (SEQ ID NO. 167) were used for this purpose. During the synthesis, the oligonucleotides were provided with a 5'-phosphate residue.

The genomic DNA was isolated from Arabidopsis thaliana as described (Galbiati M et al. Funct. Integr. Genomics 2000, 20 1:25-34).

The PCR amplification was carried out as follows:

80 ng genomic DNA
1x Expand Long Template PCR buffer
2.5 mM $MgCl_2$
in each case 350 µM dATP, dCTP, dGTP, dTTp
in each case 300 nM of each primer
2.5 units Expand Long Template Polymerase
in a final volume of 25 µl

The following temperature program is used:

1 cycle of 120 seconds at 94°C
35 cycles of 10 seconds at 94°C, 30 seconds at 48°C and 3 minutes at 68°C
1 cycle of 10 minutes at 68°C

The PCR product is purified by agarose gel electrophoresis, and the 1032 bp fragment is isolated by gel elution.

The vector pSun5 is digested with the restriction endonuclease EcoRV and likewise purified via agarose gel electrophoresis and obtained by gel elution.

The purified PCR product is cloned into the vector treated thus.

To verify the orientation of the promoter in the vector, a digestion with the restriction endonuclease BamHI is carried out. If this gives rise to a 628 bp fragment, the orientation is as shown in Fig. 43.

This construct is named p76.

The 35ST is obtained from pJIT 117 by digestion with the restriction endonucleases Kpn1 and SmaI.
The resulting 969 bp fragment is purified by agarose gel electrophoresis and isolated by gel elution.
The vector p76 is likewise digested with the restriction endonucleases Kpn1 and SmaI. The resulting 7276 bp fragment is purified by agarose gel electrophoresis and isolated by gel elution.
The resulting 35ST fragment is cloned into the p76 treated thus.
The resulting vector is named p76_35ST.

Isolation of Bgene (SEQ ID NO. 171) by means of PCR with genomic DNA from Lycopersicon esculentum as template.

The oligonucleotide primers BgeneFor (SEQ ID NO. 169) and BgeneRev (SEQ ID NO. 170) were used for this purpose. During the synthesis, the oligonucleotides were provided with a 5'-phosphate residue.

The genomic DNA was isolated from Lycopersicon esculentum as described (Galbiati M et al. Funct. Integr. Genomics 2000, 20 1:25-34).

The PCR amplification was carried out as follows:

80 ng genomic DNA
1x Expand Long Template PCR buffer
2.5 mM $MgCl_2$
in each case 350 µM dATP, dCTP, dGTP, dTTp
in each case 300 nM of each primer
2.5 units Expand Long Template Polymerase
in a final volume of 25 µl

The following temperature program was used:

1 cycle of 120 seconds at 94°C
35 cycles of 10 seconds at 94°C, 30 seconds at 48°C and 3 minutes at 68°C
1 cycle of 10 minutes at 68°C

The PCR product was purified by agarose gel electrophoresis, and the 1665 bp fragment was isolated by gel elution.

The vector p76_35ST is digested with the restriction endonuclease SmaI and likewise purified via agarose gel electrophoresis and obtained by gel elution.

The purified PCR product is cloned into the vector treated thus. To verify the orientation of Bgene in the vector, a digestion with the restriction endonuclease EcoRI is carried out. If this gives rise to a 2216 bp fragment, the orientation is as shown in Fig. 43.
This construct is named pB.
pB is digested with the restriction endonucleases PmeI and SspI, and the 3906 bp fragment comprising the promoter P76, Bgene and the 35ST is purified by agarose gel electrophoresis and obtained by gel elution.

MSP108 (Example 21, Fig.25) is digested with the restriction endonuclease Ecl126II, purified by agarose gel electrophoresis and obtained by gel elution.

The purified 3906 bp fragment comprising the promoter P76, Bgene and the 35ST from pB is cloned into the vector MSP108 which has been treated thus.

The orientation of the insert is established by restriction digestion with NcoI. If this gives rise to a fragment 5268 bp in size, the orientation is as shown in Fig. XX.
This construct is named pMKP1 (Fig. 44).

Example 38:
Preparation of expression vectors for the flower-specific expression of the chromoplast-specific lycopene beta-cyclase from Lycopersicon esculentum under the control of the promoter P76, for the flower-specific expression of the ketolase NP196 from *Nostoc punctiforme ATCC 29133* under the control of the EPSPS promoter and for the flower-specific production of dsRNA transcripts comprising 5'-terminal fragments of the epsilon-cyclase cDNA (AF251016) under the control of the AP3P promoter

Vector cspl (Fig. 34, Example 30) is digested with Ecl136II, purified by means of agarose gel electrophoresis and obtained by gel elution.

The 3906 bp SspI/PmeI fragment comprising the promoter P76, Bgene and the 35ST from pB (see Example 37) is cloned into the vector cspl which has been thus treated.

The orientatation of the insert is established by restriction digestion with SacI. If this gives rise to a fragment 3170 bp in size, the orientation is as shown in Fig. XX.

This construct is named pMKP2 (Fig. 44).

Example 39:
Generation and characterization of transgenic Tagetes plants

Tagetes plants were transformed and regenerated as described in Example 7, using nucleic acid constructs of Examples 30 to 38.

The transgenic Tagetes plants are characterized as described in Examples 8 and 9 and in Example 17.